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VOLUME 104

ACTA PHYSIOLOGICA SCANDINAVICA

Vol. 104. INDEX

Fasc. 1. (September 1978)

Fasc. 2. *(October 1978)*

Short Communications.

Fasc. 3. (November 1978)

Short Communications

Fasc. 4. (December 1978)

INDEX AUCTORUM

Acta physiol. scand. 1978. 104. 1–2



YNGVE ZOTTERMAN 80 years

On the 20th of September 1978 YNGVE ZOTTERMAN reaches the age of 80 years. It is with deep feelings of appreciation and gratitude that Acta Physiologica Scandinavica and all those associated with the Journal offer him their warmest wishes for continued happy years and successful activities. YNGVE ZOTTERMAN has the rare privilege to look back this jubilee day on a rich and varied life while still enjoying physical and spiritual health which is only granted a few.

As National Editor for Sweden of Acta Physiologica Scandinavica YNGVE ZOTTERMAN has made invaluable contributions to the development of the Journal. These are not only based on a wide experience in many sectors of physiology but also on his vivid desire to make the Journal representative for Scandinavian Physiology. His own experimental skill combined with an extensive personal knowledge of contemporary physiologists has created the best possible basis for this endeavour. His contacts with a number of leading physiologists has no doubt been stimulated by the close relationship with J. E. Johansson who played an important role in the international organization of physiology and who was once Alfred Nobel's personal assistant in biological research.

YNGVE ZOTTERMAN showed from early years unusual gifts for writing and in parallel with this also for judging the results of other people's scientific production. His own vivid and

lucid style contrasted at times markedly with scientific literature of more conventional typ but his enthusiasm was readily aroused when reading papers revealing original ideas, ne aspects and significant discoveries.

This spiritual background made YNGVE ZOTTERMAN singularly well prepared for the responsibilities connected with Editorship for a scientific Journal. His judgement as selection of suitable referees has been of paramount value for the Journal.

In his capacity as National Editor for this journal he also has had many opportunities to give valuable and stimulating advice to younger authors thereby giving them a lead to new and fruitful aspects of the problem. Sometimes he helped to refresh the memory of more experienced authors as regards the historical development of the problem studied. His interest in the history of science has, as time has passed, led to an accumulation of knowledge in this area which is probably unique in our days. On many occasions his friends and colleagues have enjoyed this invaluable source of—often amusing—information. Many of these treasures have been saved for the future in his book with the witty title "Touch, Tickle and Pain"

YNGVE ZOTTERMAN's contributions to our journal is indeed not restricted to his Editorship however valuable this is and has been. His contributions as author of articles of highest significance spans over a period of some 50 years. In a remarkable series of papers he has covered a number of areas and produced results which have forever connected his name with such fundamental fields as the physiology of single nerve fibres (thesis 1933), the neurophysiology and neurochemistry of the carotid sinus and the carotid body, the physiology of taste, to mention some of the most important ones, each of which would secure him a place in contemporary physiology.

It has been a great privilege for this journal to publish these articles. It is through contributions of this caliber that the reputation of a journal is founded and maintained. To this should be added his outstanding work as organizer of work of vital importance for national nutrition, old age care and his successful work for scientific international symposia will not fail to appear in other contexts. In his indefatigable efforts for the furthering of Scandinavian Physiology the unfailing support from his charming wife should not be forgotten.

On his birthday YNGVE ZOTTERMAN will receive congratulations from all parts of the world. It is with pride and admiration that the Scandinavian Physiologists, through their official publication, add their warm wishes to a great physiologist and friend and express their gratitude for his invaluable work for Scandinavian Physiology.

U. S. von Euler

Acta physiol. scand. 1978. 104. 3–12

From the Institute of Neurobiology and Department of Surgery III, University of Göteborg, Sweden

Ultrastructural evidence for an innervation of epithelial enterochromaffine cells in the guinea pig duodenum

By

JAN M. LUNDBERG, ANNICA DAHLSTRÖM, ANDERS BYLOCK, HÅKAN AHLMAN, GÖSTA PETTERSSON, INGER LARSSON, HANS-ARNE HANSSON and JAN KEWENTER

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Abstract

LUNDBERG, J. M., A. DAHLSTRÖM, A. BYLOCK, H. AHLMAN, G. PETTERSSON, I. LARSSON, H.-A. HANSSON and J. KEWENTER. *Ultrastructural evidence for an innervation of epithelial enterochromaffine cells in the guinea pig duodenum.* Acta physiol. scand. 1978. *104* 3–12.

The innervation of the duodenal enterochromaffine cells (E.C.) of the guinea pig was studied at the electronmicroscopic level. Pretreatment with 5-OH-dopamine was performed to visualize catecholaminergic (CA) nervous elements. Near the basement membrane of all examined E.C. in the crypts, bundles of unmyelinated nerve processes were observed, only partly embedded in Schwann cell cover. At least 4 types of processes could be observed. 1) Boutons containing only small clear vesicles, probably cholinergic fibres, 2) boutons with small clear vesicles, and in addition large (200 nm) granules with dense matrix (P-type-fibres), 3) boutons with small electron-dense vesicles, probably CA-fibres; and 4) processes with few vesicles but having the appearance of dendrites. No typical synaptic arrangements were observed, but the minimal distance between the E.C. and the nerve bundles was 150 to 250 nm, thus well within the functional limits of the "autonomic gap". Thus, epithelial E.C. may be influenced by several types of nervous elements, including CA-fibres.

Key words: enterochromaffine cells, guinea pig duodenum, electron microscopy, adrenergic nerve terminals, bundles of nerve processes

The enterochromaffine cells (E.C.) in mammals, such as mouse, rat, guinea pig, cat and man, have been shown to contain serotonin (5-HT) stored in large granules in the cytoplasm (for ref. see Pearse 1969 and Ahlman 1976). In addition to 5-HT the E. C., constituting part of the entero-endocrine cell system, probably also contain some polypeptide gastrointestinal hormones as demonstrated by immunohistochemistry *e.g.* motilin or substance P (ref. Pearse *et al.* 1974, Heitz *et al.* 1976, Polak *et al.* 1976). Thus, the E.C. of the small intestine belong to the so called APUD-group of endocrine cells, which can take up amine precursors, decarboxylate them and store the amine together with the hormonal peptide (*cf.* Pearse 1969). Also, a common origin of APUD cells from neurally programmed cells of the epiblastic origin has been proposed (*cf.* Pearse *et al.* 1977). Other entero-endocrine

cells in the gut have been shown to store other polypeptides, for instance VIP gastrin or somatostatin (for ref see Pearse et al 1977)

The functional control of the endocrine cells in the gut, e.g the E.C. cells, is unknown at present, but suggestions have focused around four main possible mechanisms

1) Control by changes in the intraluminal milieu of the gut. The shape of the cell, with a thin apical portion carrying villous projections into the gut lumen, is highly suggestive of a receptor function. Instillations of hypertonic glucose and acidification of the duodenum cause degranulation of E.C. and release of 5-HT (e.g Drapanas et al 1962, Fujita and Kobayashi 1971 Kellum and Jaffe 1976)

2) Control by substances circulating in the blood stream. For instance, noradrenaline (NA) infusion to the isolated dog intestine has been shown to cause a release of 5-HT into the perfusate (Burks and Long 1966) Therefore, the suprarenals may hypothetically control the 5-HT release from the E.C. by circulating levels of catecholamines. Also alterations in the level of Ca^{++} in the blood may influence the E.C. (Jaffe, personal communication, 1977)

3) Control by hormones, locally released from neighbouring cells. For instance, gastrin releasing cells of the gastric mucosa have been suggested to be controlled by locally released somatostatin from adjacent cells (cf Uvnäs-Wallensten et al 1977)

4) Autonomic nervous control. Previous ultrastructural studies on a possible innervation of E.C. have demonstrated the presence of bundles of non myelinated nerve fibres close to the basal lamina of the E.C., but no clear identification of nerve terminal structures was made (Gasbarrini et al 1969) However fluorescence histochemical studies have given morphological support for an adrenergic innervation of E.C Thus, catecholamine (CA) containing varicose fibres have been observed near E.C. in the intestinal crypts (Jacobowitz 1965 Gabella and Costa 1968 Ahlman et al 1976 a). Also functional evidence for a nervous control of duodenal E.C. has been obtained. Nerve stimulation of the cervical trunk of the vagal nerve caused a decrease in 5-HT content of the duodenum of guinea pig (Hobenleitner et al 1971) This decrease in 5-HT could be directly correlated to the E C. in a cytofluorimetrical study which demonstrated a clear decrease in 5-HT content in individual E.C. following stimulation of the cervical vagal nerve in cat (Ahlman et al 1976 a). Furthermore, denervation experiments and pretreatment with adrenergic blocking agents gave results which strongly suggested that an adrenergic neuronal pathway was involved (Ahlman et al 1976 b). This is in agreement with previous investigations which reported a release of 5-HT from the small intestine upon stimulation of periarterial sympathetic nerves or intra-arterial infusion of noradrenaline (NA) (Burks and Long 1966, Tansy et al 1971)

Thus, both light microscopical and functional support for a nervous adrenergic control of the duodenal E.C. exists. The purpose of the present study was to investigate the possible presence of a direct innervation of duodenal E.C. with particular respect to adrenergic nerves.

Material and methods

In order to identify adrenergic nerve terminals of the guinea pig duodenum, 5-hydroxydopamine (5-OHDA) pretreatment was performed (Tranzer and Thoenen 1967)). 1 h or 24 h after the i.v injection of 2

of 5-OH-DA (Ciba 1973), the guinea pigs were anesthetized with Mebumal sodium, 30 mg/kg i.p., and perfusion-fixed with 2.5% purified glutaraldehyde in 0.15 M cacodylate buffer pH 7.2, at 20°C. The proximal duodenum was then dissected open and immersion-fixed in the same solution for 2 h at 4°C. The tissues of a few animals were immersion-fixed only. Thereafter small blocks of the tissues were post-fixed for 90 min in a buffered solution of 2% osmium tetroxide. The fixed tissue blocks were then dehydrated in a graded series of ethanol and embedded in Epon®. Semi-thin (1 µm) sections were cut to localize the E.C. in the basal portions of the crypts, based on their content of large irregular osmiophilic granules (e.g. Nichols *et al.* 1974). Thereafter, ultrathin serial sections were made of the appropriate areas and contrasted with uranyl acetate and lead citrate.

Results

The E.C. were easily observed in the electron microscope due to the presence in the cytoplasm of a large number of irregular granules with a matrix of varying electron-density (*cf* Ferreira 1971, Nichols *et al.* 1974, Cheng and Leblond 1974). These granules were in many E.C. very numerous in the basal part of the cell, but also many E.C. with mainly apically situated granules were observed (Fig. 1). The basal parts of the cells rested on the basement membrane of the crypt epithelium (Fig. 2 and 3) while their apices were often extending into the lumen by villous projections (Fig. 1 and 2). Close to the basement membrane bundles of unmyelinated nerve fibres, incompletely ensheathed in a Schwann's cell cover, were frequently observed (Fig. 2 to 5). Observations of serial sections revealed that every examined E.C. cell, and sometimes also neighbouring epithelial cells (Fig. 2), was accompanied by one or more such bundles of nerve fibres. A frequent observation was that the Schwann cell cover was often discontinuous near the base of the E.C. but completely ensheathing the nerve bundles near other intestinal cells (Fig. 2). In many cases typical boutons, filled with apparent synaptic vesicles, were seen close to the basement membrane of E.C. (Fig. 2, 3 and 5). At such places, the Schwann cell cover in most cases was missing (Fig. 3 and 5). The majority of these boutons were filled with clear round vesicles, about 40–45 nm diam. However, one or two such boutons in nerve fibre bundles close to the E.C. basement membrane were filled with dark osmiophilic granules (about 45 nm diam.), probably due to the uptake of 5-OH DA in adrenergic nerve terminals (Fig. 1 to 3). No typical synaptic structures could be observed in relation to the boutons near the basement membrane, but the minimum distance between such boutons and the basal membrane of E.C. was 150 to 250 nm (Fig. 3, 5). In some cases, P-type nerve boutons (possibly storing a polypeptide, *cf* Baumgarten *et al.* 1970) with large dense vesicles (about 200 nm diam.) were present in the bundles of nerve fibres (Fig. 4).

In the bundles of nerve processes near the E.C. also a vesicle-poor type of fibre was seen (Fig. 4). These processes were of varying diameter, but often quite large (2–4 µm). Their cytoplasm contained a few clear vesicles, filaments, microtubules and some mitochondria, but no ribosomes (Fig. 1 and 4). Their appearance was similar to that of dendritic processes observed in the inferior mesenteric ganglion of the cat (Elfvin 1971). In some nerve processes dense material, distributed in a patchlike manner, was present just inside the part of membrane which was close to an E.C. Near such membrane specializations, an aggregation of small vesicles was observed (Fig. 4 and 5).

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Discussion

The group entero-endocrine (E.E.) cells" in the small intestine contain cells with electrondense granules of varying shape and size, and which store different polypeptide hormones (e.g. review Pearse et al. 1977). In the duodenum of most mammals, the majority of E.E. cells are argentaffine (>90%), probably due their content of 5-HT (cf Nichols et al. 1974). Only 5-HT containing E.E. cells are referred to as E.C. cells (cf Pearse et al. 1977). These cells, when studied by immunocytochemical methods, appear to contain in addition to 5-HT also substance P or motilin (see ref. in Pearse et al. 1977). E.E. cells containing somatostatin, VIP or neurotensin do not appear to store 5-HT (e.g. Sandler et al. 1977 Polak et al. 1976), and are mostly distributed in lower parts of the gastrointestinal tracts than the duodenum (Polak et al 1976). A few neurotensin cells were, however, observed in the guinea pig duodenum, but only in the villous mucosa, never in the crypts. Thus, the E.E. cells in the crypts of the guinea pig duodenum with large irregular granules, studied in this investigation, are most likely true E.C. cells. Gorgas and Böck (1976) describe various types of granules in E.C. situated in villi and in crypts of the guinea pig duodenum. It is supporting our view that we have studied the E.C. cells that the granules in cryptal E.C. cells were large and irregular (as in our study) rather than small, ovoid or dumb-bell shaped (Gorgas and Böck 1976). Therefore, based on electronmicroscopical characteristics only without having performed any chromaffine or argentaffine reaction, we consider our cells to represent E.C. cells.

The method to demonstrate catecholaminergic nerve terminals by the 5-OH DA-method according to Tranzer and Thoenen (1967) has been evaluated thoroughly by e.g. Chiba (1973). According to this author the administration of more than 100 mg/kg 5-OH-DA given i.v. or i.p. 1–24 h before death results in a labelling of most adrenergic nerve terminals and of up to 90% of all vesicles in a particular nerve ending. In our studies there as a marked labelling of nerve terminals around blood vessels, indicating that the 5-OH-DA method was working satisfactory in the present experiments. The observation that nerve terminal profiles, labelled with dense cored vesicles, were present in nerve fibre bundles near the basal part of E.C. thus indicates that catecholamine-containing nerve-terminals

Fig. 1 An enterochromaffine cell (EC) from the duodenum of 5-OH-DA-pretreated guinea-pig. The EC contains abundant irregular granules, situated both in the basal part, and in the apical region. Villous projections from the apex of the EC into the gut lumen can be observed (▶). Close to the base of the EC bundle of nerve processes (N) is situated. One contains electron dense granules (probably due to uptake of 5-OH DA) and presumably representing catecholaminergic nerve terminal (→). Other fibres contain small clear vesicles (45 nm diam.). A large vesicle-poor process is also seen, with the appearance of dendritic process (→) (Bar represents 1 μm).

Fig. Oblique section through bundle of nerve processes close to the base of guinea-pig enterochromaffine cell (EC), containing some basal granules. BL = basal lamina of the epithelium. S = Schwann cell cytoplasm which is discontinuous near the base of the EC. IE = intestinal epithelial cell also bordering the nerve bundle. In the nerve bundle both presumably catecholaminergic (→ stained by the 5-OH-DA pretreatment), possibly cholinergic (→ containing small clear vesicles and few larger dense core vesicles, diam. 70–90 nm) and vesicle-poor dendrite-like (D) nerve endings are present (Bar represents 1 μm).

Fig. 3 The basal part of guinea-pig EC (EC) with dense, irregular granules. BL = the basal lamina of the epithelium. A bundle of nerve fibres is present close to the base of the EC. Both 5-OH-DA-labelled catecholaminergic nerves (→) and profiles with small clear vesicles (▶) are present. Note that Schwann cell lamina (S) is discontinuous near the EC. MT = microtubules. (Bar represents 1 μm).

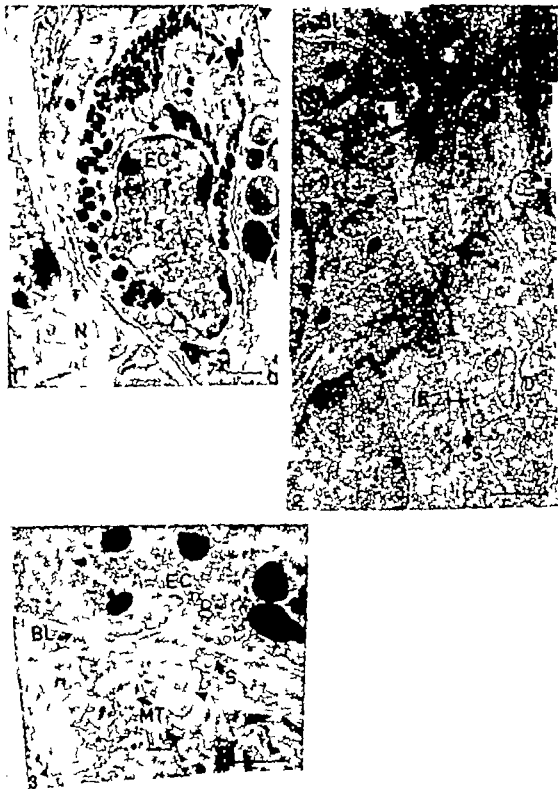


Fig. 1 3

were present near the basement membrane of E.C. Other types of nerve fibres, containing small (45–50 nm) clear vesicles, alone or together with large granules (about 200 nm) with dense matrix (P-type, *cf* Baumgarten *et al.*, 1972) were also present close to the E.C.

Innervation of surface-lining epithelial cells has been described to be present also in other organs, *e.g.* the gall bladder and the stomach. The gall-bladder epithelium, which has a secretory function, is densely innervated by autonomic nerve fibres showing the charac teristics of cholinergic nerve-endings. Typical synaptic arrangements were searched for in the study but not observed electronmicroscopically. However a functional effect of cholinomimetics on the surface epithelial cells could be demonstrated, suggesting the presence of acetylcholine receptors on the epithelial cells and thus also a nervous control (Axelsson *et al.* 1977). In the stomach, the parietal cells are innervated by cholinergic fibres (*cf* Hankert *et al.* 1977). In the intestine, the secreting Paneth s cells have been shown to be innervated by both adrenergic (Gabella and Costa 1968) and cholinergic (*cf* Ahonen 1973) nerve fibres (also, Lundberg *et al.*, unpublished observations). The E.C. cell, which is also a secretory cell, may thus, in analogy with the gall bladder secretory cells, the parietal cells of the ventricle, and the Paneth s cells, be expected to be innervated by efferent fibres. Since the epithelial E.C. is also an APUD-cell (*cf* Pearse 1969), and since other APUD-cells, *e.g.* pancreatic islet cells (Coupland 1958, Libman and Sutherland 1965, Cegrell 1968), and submucosal endocrine cells (Matsuo and Seki 1976) receive an autonomic innervation, it is not surprising to find morphological evidence for an innervation of the epithelial E.C. (*cf* also Gabbarrini *et al.* 1969).

In the nerve fibre bundles close to the E.C., nerve processes, essentially devoid of synaptic vesicles, were also present (Fig. 4). These large processes, containing mitochondria, occasional sER-profiles and a few filaments and tubules, might represent dendritic processes, because they have many of the morphological characteristics of dendrites, as described by *e.g.* Elfvin (1971). No ribosomes could be observed in these processes, but if they represent distal parts of dendrites, one would not expect to see many ribosomes. In dendritic processes closer to the cell body ribosomes are observed more frequently (see Taxi 1974). In some cases, such processes contained dense patches attached to the neuronal membrane (Fig. 4, 5). Near such a region a collection of vesicular profiles was often present (Fig. 5). Such membrane patches with nearby vesicles have been observed previously in dendritic processes of superior cervical ganglion cells (*cf* Taxi 1974).

The presence of possible dendritic processes near E.C., suggesting an afferent innerva-

Fig. 4. The basal part of a guinea-pig EC (EC) with many irregular electron dense granules. Near the EC a bundle of nerve processes, many of which contain a few large (diam. about 200 nm) electron dense granules with a light halo. These nerve processes have the appearance of P-type nerves, possibly storing polypeptide. A large dendrite-like process, with mitochondria but very few vesicles, is also present (D). Some membrane specializations can be seen in this process (→). MT = microtubules. (Bar represents 1 μm)

Fig. 5. The basal part of a guinea-pig EC (EC) with basal granules and ribosomes. N indicates a nerve process, filled with small clear vesicles and some sER-profiles (▶). Membrane specializations can be observed as 2 patches of electron dense material attached to the inner membrane (→). No Schwann cell lamina present. In the EC two large clear vesicles (coated), which may represent endo- or exocytotic vesicles can be seen (↔). The distance between the nerve process and the EC is 150–200 nm. (Bar represents 0.5 μm).



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Figs. 4 and 5

E.C. An *indirect* influence acting via an inhibitory interneuron, is also possible. This hypothetical non-adrenergic interneuron is probably not cholinergic, because atropine did not influence 5-HT release from E.C. in *in vivo* experiments (Ahlman *et al.* 1976 b).

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tion, is very interesting from a functional point of view Thus, the postprandial vasodilatation in the duodenal mucosa, which can be elicited experimentally by mechanical mucosal stimulation (*cf* Biber *et al* 1971), probably activates an intramural neuronal arch, involving 5-HT as a synaptic transmitter (*cf* Biber 1973 p 21) The epithelial E.C., having villous processes projecting into the gut lumen, may respond not only to glucose installations and a changed pH in the gut lumen (*cf* Fujita and Kobayashi 1971 Resnick and Gray 1962), but also to mechanical stimulation. This may lead to a liberation of 5-HT and also of the polypeptide (substance P or motilin) which is presumably stored together with 5-HT (*cf* Pearse *et al* 1974 1977) in the large osmiophilic, cytoplasmic granules in the basal end of the E.C., in the same way as calcitonin is stored with 5-HT in sheep parafollicular granules (Atack *et al* 1973) The 5-HT could then possibly activate the suggested dendritic processes, observed in this study in accordance with the arrangement suggested by Biber (1973) It is of interest in this connection to mention tha sensory neurons (type II neurons according to Dogiel 1896) of the submucous and myenteric plexuses of the small intestine contain a monoamine oxidase specific for tryptamines (Furness and Costa 1971) The polypeptide, which is presumably released together with 5-HT may of course, also influence dendritic nervous elements, but it may also act as a paraendocrine substance, influencing nearby cell types (*cf* Uvnäs-Wallensten *et al* 1977)

The origin of the various nervous elements observed near E.C. in the present study is still obscure. As concerns the *efferent* systems, the adrenergic nerve terminals presumably originate from adrenergic cell bodies situated outside the gut *e.g* the celiac and/or the mesenteric ganglia. Some of these fibres may also originate in the superior cervical ganglia, since there exist both functional and morphological evidence for a descending adrenergic neuronal pathway from the superior cervical ganglia via the vagal nerves to the duodenal mucosa (*cf* Ahlman *et al* 1976, Lundberg *et al* 1976) The cholinergic nerve fibres may originate from either the submucosal plexus or from the efferent fibres in the vagal nerve. The P type axons, finally may be processes from polypeptide storing nerve cell bodies which have been observed using immunohistochemical methods in both the *plexus submucosus* and the *plexus myentericus* or from peptide storing neurons in the spinal ganglia (*cf* Hökfelt *et al* 1977) The possible *afferent* nerve processes may hypothetically originate from sensory neurons in the nodose ganglia or possibly from nerve cells in the intramural plexa. According to Dogiel (1896) two types of neurons are present in the submucous plexus. One is probably a motor neuron with one long axon and several short dendrites. The other one (Type II) is probably a sensory neuron with long branched dendrites, ending as sensory receptors. The presumably dendritic processes observed near E.C. in the present study may originate from such sensory cells of the submucous or myenteric plexuses. These sensory cells appear to receive a direct adrenergic innervation while motor cells receive a parasympathetic (probably cholinergic) input (*cf* Jabonero 1953).

The previously demonstrated adrenergic release of 5-HT from E.C., elicited by stimulation of descending CA-fibres in the cervical vagal nerve (Ahlman *et al* 1976 a) may operate by direct or indirect nervous influence of the E.C. The *direct influence* appears to be possible from a morphological point of view since adrenergic nerve terminals have been demonstrated to be present in nerve bundles close to the basal lamina of epithelial

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From the Institute of Physiology University of Bergen, Norway

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Received 2 January 1978

Abstract

Tønder, K. H. and G. Næss. *Nervous control of blood flow in the dental pulp in dogs.* Acta physiol. scand. 1978. *104* 13–23

Pulpal blood flow (PBF) was measured by H_2-gas clearance in 30 dogs during electrical nerve stimulation, denervation and close intraarterial infusion of noradrenaline (NA). Electrical stimulation of the cervical sympathetic nerve and NA infusion always resulted in decreased PBF. The constrictor effect was reduced by α-blockers, but was never abolished or reversed to vasodilation. Thus, no evidence for β-receptors in the dental pulp blood vessels was found. The fall in PBF produced by cervical sympathetic stimulation could be prevented by cutting the inferior alveolar nerve in the mandibular canal. Stimulation of the cut inferior alveolar nerve in the mandibular canal produced variable pulpal flow changes. Both marked increase and decrease were measured. The flow changes during inferior nerve stimulation were unaffected by α-receptor blockade, while atropine reduced the vasoconstrictor response or augmented the dilatory response. Electrical stimulation of the mandibular nerve before its entrance in the canal caused vasodilation, proposing a sensory antidromic axonreflex in the dental pulp. Sympathetic denervation had little or no effect on PBF while cutting of the inferior alveolar nerve in the canal increased PBF in lower canines from 0.159 to 0.219 ml/min g. The experiments show that pulp blood vessels of the dog may be controlled by both vasodilator and vasoconstrictor nerve fibers.

Despite the abundant histological evidence for the presence of different nerve fibers terminating on or in close contact with dental pulp vessels, little information is available on the vaso-regulatory function of these fibers. Arwill (1958) found two types of unmyelinated nerve fibers in the human dental pulp, but only one of them was supposed to be of autonomic origin and located in the vascular wall. A close connection of sympathetic fibers with pulpal vessels and an acetyl-cholinesterase-positive nerve fibre network on some blood vessels have been demonstrated by Pohto and Antila (1968).

Avery *et al.* (1971) noted a significant drop in specific cholinesterase after resection of the inferior alveolar nerve, whereas removal of the cervical sympathetic nerve resulted in an increased cholinesterase reaction throughout the pulp. The presence of cholinergic nerve fibers in the pulp has also been supported by Avery *et al.* (1974) and Kukletová *et al.* (1968).

A number of investigators have concluded that stimulation of the cervical sympathetic nerve or infusion of catecholamines caused a reduction of blood flow in the dental pulp (Taylor 1950, Ogilvie 1969, Edwall and Kindlová 1971, Beer *et al.* (1974), but information concerning the type of adrenergic receptors is sparse and partly contradictory (Weiss

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Pulpal blood flow (PBF) was measured by H_2-gas clearance in 30 dogs during electrical nerve stimulation, denervation and close intraarterial infusion of noradrenaline (NA). Electrical stimulation of the cervical sympathetic nerve and NA infusion always resulted in decreased PBF. The constrictor effect was reduced by α-blockers, but was never abolished or reversed to vasodilatation. Thus, no evidence for β-receptors in the dental pulp blood vessels was found. The fall in PBF produced by cervical sympathetic stimulation could be prevented by cutting the inferior alveolar nerve in the mandibular canal. Stimulation of the cut inferior alveolar nerve in the mandibular canal produced variable pulpal flow changes: Both marked increases and decreases were measured. The flow changes during inferior nerve stimulation were unaffected by β-receptor blockade while atropine reduced the vasoconstrictor response or augmented the dilatory response. Electrical stimulation of the mandibular nerve before its entrance in the canal caused vasodilatation, proposing a sensory antidromic axonreflex in the dental pulp. Sympathetic denervation had little or no effect on PBF, while cutting of the inferior alveolar nerve in the canal increased PBF in lower canine from 0.159 to 0.219 ml/min g. The experiments show that pulp blood vessels of the dog may be controlled by both vasodilator and vasoconstrictor nerve fibers.

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A number of investigators have concluded that stimulation of the cervical sympathetic nerve or infusion of catecholamines caused a reduction of blood flow in the dental pulp (Taylor 1950, Ogilvie 1969, Edwall and Kindlova 1971, Beer *et al.* (1974), but information concerning the type of adrenergic receptors is sparse and partly contradictory (Weiss

et al 1969 Edwall 1971 Boivin *et al* 1974) The sympathetic postganglionic fibers arising in the superior cervical ganglion reach the dental pulp of the lower teeth by travelling as a plexus around the carotid artery through the mandibular nerve and finally through the inferior alveolar nerve (Christensen 1940, Ogilvie 1969) However previous studies of stimulation of the mandibular or inferior alveolar nerve have given conflicting results in regard of blood flow Thus Kroeger (1968) stimulated the distal cut end of the inferior alveolar nerve and recorded an increase in intrapulpal pressure (opposite of the response to sympathetic stimulation) while stimulation of the mandibular nerve in the canal produced no change in pulpal blood flow measured by impedance plethysmography (Neidle and Liebman 1964) Bishop and Dorman (1963) reported that electrical stimulation of the distal end of the cut mandibular nerve produced vasoconstriction, while sectioning of the mandibular nerve resulted in an increased blood flow in the mandibular artery This increase in flow might result from removal of sympathetic vascular basal tone or mechanical stimulation of vasodilator fibers. However experimental evidence for nervous vasodilation in the pulp caused by either sympathetic or other nerve fibers is lacking.

The purpose of the present investigation was to study pulpal blood flow changes during electrical nerve stimulation and denervation in order to determine whether the various nerves contribute vasomotor efferent fibers, either dilator or constrictor to the blood vessels in the dental pulp in dogs. The cervical sympathetic, mandibular and inferior alveolar nerves were stimulated and attempts were made to identify the vasoactive fibers by means of receptor blocking drugs.

Methods

Experiments were performed on 30 adult healthy mongrel dogs of different sizes and sex. The animals were anesthetized by intravenous injection of Nembutal (sodium pentobarbital), 25 mg per kg b.w A tracheal cannula was inserted and the jaw immobilized. A femoral artery and vein were exposed and catheterized, the vei for infusion and the artery for continuous recording of systemic rterial pressure with a Hewlett Packard transducer and recorder

Blood flow in the dental pulp of the canine teeth was estimated by measuring local H_2-gas clearance with platinum electrodes implanted in th pulp 2–8 day previously (Tønder and Aukland 1975). H_2-gas was supplied by inhalation of about 5% H_2 air for 5–15 min, providing stable concentration in the pulp. Pulpal blood flow (PBF) in ml/min per g tissue was calculated from the rat constant of the H desaturation curves obtained after stopping H_2-gas inhalation. The rate constant, k, was obtained by plotting the electrode current caused by oxidation of H_2(I_{H_2}) on a logarithmic scale against linear time scale as previously described (Tønder 76). The coefficient of variation for subsequent measurements was with this method 3 1% (Tønder and Aukland 1975).

The cervical sympathetic, the mandibular or the inferior alveolar nerve was dissected free for electrical stimulation on the side where pulpal blood flow was measured. In experiments with sympathetic stimulation the common carotid artery was exposed together with the sympathetic nerve, and blood flow the common carotid artery (CCBF) was recorded with a Nycotron electromagnetic flowmeter The stimulating electrode was placed caudal t the superior cervical ganglion and preganglionic stimulation was performed.

The sympathetic nerve in the neck of the dog runs together with the vagus in common vago-sympathetic trunk and careful dissection of the trunk was necessary t separate the ympathetic portion. Identification of the sympathetic nerve was made functionally by observing the ipsilateral pupil and blood flow in the common carotid during stimulation. The sympathetic nerve was cut proximal to the stimulation point to exclude spreading of the stimulus via afferent fibers.

The mandibular nerve was exposed through intraoral incision, dissected free and cut one cm before its entry into the mandibular foramen. The inferior alveolar nerve was surgically exposed in the mand bular

canal by removal of a buccal segment of cortical bone distal to the second premolar. All nerves were stimulated in efferent direction using a bipolar silver wire electrode with monophasic square wave pulses (Grass Model). The parameters of the stimulation were: pulse duration 0.5–6 ms, frequency 1–20 Hz and voltage 4–30 V. The stimulation periods were of 4 to 12 min duration.

In those experiments where the effect of noradrenaline (NA) was tested the external carotid artery was dissected free through an incision behind the jaws on the side of the examined teeth. The lingual artery was cannulated in retrograde direction for NA infusion. An electromagnetic flow probe (Nycotron, Oslo) with diameter 2.5 mm was placed on the external carotid a few mm distal to the infusion site.

The following drugs were used: Noradrenaline (0.01–5 µg/min), atropine (sulphate 1 mg/min) and phenoxybenzamine (30 µg/min). The drugs were diluted in 0.9% saline and infused at a rate of 1 ml/min. PBF was measured during control conditions alternating with periods of nerve stimulation and/or drug infusion.

Pulpal vascular resistance (R) was calculated as arterial pressure in mmHg divided by PBF in ml/min g, giving the resistance unit mmHg min g/ml. For comparison of different experiments, the values of PBF, AP and R were calculated in per cent of the averaged control values obtained immediately before and after the experimental periods.

Results

Control measurements

Control measurements were usually made before and after stimulation and drug infusion, and their average was used for comparison with the intervening experimental period. Mean PBF measured in the canine teeth in 30 dogs during control conditions was 0.166 (SE 0.003) ml/min g (83 measurements) and control systemic pressure averaged 115 mmHg. Usually arterial pressure and pulpal blood flow remained fairly constant even in experiments of several hours' duration.

Effect of cutting the inferior alveolar nerve

Measurements of PBF in the lower canine were performed before and after cutting the ipsilateral inferior alveolar nerve in the canal in 13 dogs. Compared to recordings after surgical exposure of the nerve, denervation caused a rise in pulpal blood flow of ipsilateral lower canine in 10 dogs. In three dogs, cutting of the nerve had no effect on PBF (Fig. 1). Control measurements before surgical exposure of the nerve (5 dogs) demonstrated that surgery had no consistent effect on PBF. On average, PBF in the ipsilateral lower canine rose from 0.159 (SE 0.017) ml/min g to 0.219 (SE 0.027) ml/min g after denervation. Calculated pulpal resistance fell on average by 23 per cent, but repeated measurements during the first hours after denervation showed that this fall in resistance might be transient (Fig. 1). Simultaneous recordings of PBF in the ipsilateral upper canine showed no consistent change after cutting the inferior alveolar nerve.

Inferior alveolar nerve stimulation

Efferent stimulation of the cut inferior alveolar nerve in the canal had inconsistent effect on blood flow in the ipsilateral lower canine, as shown in Fig. 2 (left panel). Stimulation caused no change in systemic arterial pressure, while PBF ranged from 35–153 per cent compared to control measurements. In a total of 27 measurements (16 dogs), PBF fell to 91% (SE 6) of control and calculated vascular resistance rose to an average of 121% (SE 8) of control (Fig. 3, left panel). Simultaneous measurements of PBF in the ipsilateral

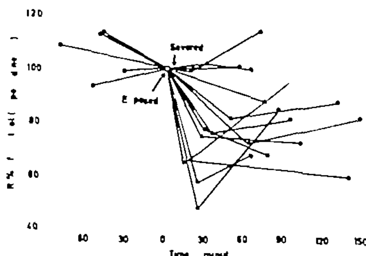


Fig. 1 Effect of cutting the inferior alveolar nerve on pulpal vascular resistance (R) in ipsilateral lower canine tooth. — Open circles: Control measurements. Closed triangles: First measurements after denervation. Closed circles: Second measurements after denervation.

upper canines (12 dogs) showed that blood flow here remained unaltered during the stimulation periods.

In 7 dogs (9 measurements) phenoxybenzamine (7 mg/kg b w) was given i.v prior to stimulation. As shown in Fig. 3 right panel, alpha-adrenergic blockade did not significantly change the effect of stimulation of the inferior alveolar nerve. Control blood flow in the lower canine averaged 0 156 (SE 0 013) ml/min g compared to 0 165 (SE 0 022) ml/min g *during stimulation and calculated pulpal resistance showed no consistent change* ($p < 0.05$)

Intravenous infusion of atropine in 6 dogs consistently altered the effect of stimulation on PBF in the ipsilateral lower canine. Compared to measurements during nerve stimulation before atropine administration blood flow in the lower canine was higher during stimulation after atropine infusion This was a surprising but consistent finding in recordings in 6 dogs. Thus, in all 6 expts. pulpal vascular resistance (R) was lower during stimulation after (77% of controls) than before (115% of controls) infusion of atropine (Fig. 4). Atropine had no effect on simultaneously recorded PBF in the unstimulated ipsilateral upper canine tooth, or during control measurements.

Cervical sympathetic stimulation

When the cervical sympathetic nerve was stimulated, a decrease in common carotid blood flow (CCBF) and PBF in the ipsilateral lower and upper canines was recorded in all experiments, as exemplified in Fig. 5 A moderate rise in systemic blood pressure was noted in some experiments. The fall in pulpal blood flow was partly blocked by phenoxybenzamine, whereas atropine had no effect (Fig. 5) In a total of 25 measurements (12 dogs), PBF fell on average to 35% of control (Fig. 2, middle panel), and calculated pulpal resistance rose by 50 to 1 000%. Section of the sympathetic nerve caused no measurable change in PBF whereas CCBF rose on average 50%.

Effect of cutting the inferior alveolar nerve during cervical sympathetic stimulation

To test whether the pulpal vasoconstrictor fibers from the cervical sympathetic nerve run in the inferior alveolar nerve in the mandibular canal, the inferior alveolar nerve was cut

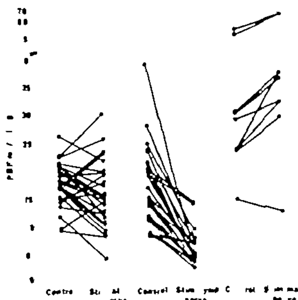


Fig. 2. Pulpal blood flow (PBF) during electrical stimulation of the alveolar inferior nerve (left panel), the cervical sympathetic nerve (middle panel) and the mandibular nerve (right panel). Control measurements are indicated by open circles.

during cervical sympathetic stimulation. Typical responses are illustrated in Fig. 6. Nerve section had no effect on PBF in the ipsilateral upper canine tooth, whereas PBF in the lower canine abruptly increased inspite of continued sympathetic stimulation. Repeated sympathetic stimulation after cutting the inferior alveolar nerve gave a significant fall in PBF in upper canine, while PBF in lower canine was unchanged compared to control measurements (Fig. 6). Similar effects were obtained in 3 additional dogs. Systemic arterial pressure and CCBF were not affected by the denervation. As described above (Fig. 2), subsequent stimulation of the cut distal end of the inferior alveolar nerve had an inconsistent effect on PBF in the ipsilateral lower canine (Fig. 6).

The average fall in PBF during sympathetic stimulation before nerve section was 69% (4 dogs), whereas stimulation of the cut distal end of the inferior alveolar nerve caused a mean rise in PBF of 16% of controls in the same animals.

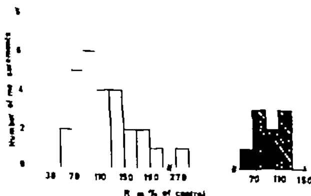


Fig. 3. Pulpal vascular resistance (R) in lower canine tooth during efferent stimulation of the cut inferior alveolar nerve. Left panel: Before phenoxybenzamine. Right panel: After phenoxybenzamine, 7 mg/kg b.w.

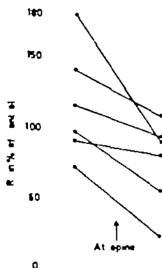


Fig. 4

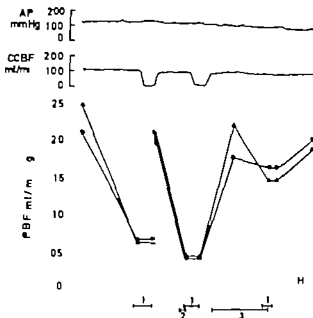


Fig. 5

Fig. 4. Pulpal vascular resistance (R) in per cent of control during inferior alveolar nerve stimulation (6 dogs). Open circles before, and closed circles, after atropine (0.3 mg/kg b.w.) administration.

Fig. 5 Simultaneous recordings of systemic arterial pressure (AP), common carotid blood flow (CCBF) and pulpal blood flow (PBF) in ipsilateral upper (closed triangles) and lower (closed circles) canine tooth. 1 Cervical sympathetic stimulation 6 V 2 ms, 6 Hz, 2. I.v infusion of atropine, 0.3 mg/kg b.w 3 I v i fusion of phenoxybenzamine 50 μg/min.

Mandibular nerve stimulation

The effect on PBF by efferent stimulation of the cut mandibular nerve before its entrance into the mandibular canal was studied in 5 dogs (9 measurements). During stimulation PBF in ipsilateral lower canine increased in all but one measurement (Fig. 2, right panel), on average by 27%. Systemic arterial pressure remained unchanged

The majority of the present recordings during the various nerve stimulations were made with stimulus frequencies of 5 to 10 Hz. Frequency ranges of 1–5 or 10–20 Hz were used in only a few experiments. While these frequences never revealed qualitatively different responses, the observations are too few to establish acceptable frequency/response curves.

Close intra-arterial infusion of noradrenalin

Close intra-arterial infusion of NA (0 01–5 μg/min) always caused a fall in external carotid blood flow (ECBF) and PBF (8 dogs/70 measurements), as exemplified in Fig. 7 The vasoconstriction induced by NA infusion was only partly blocked by α-blockers (Fig. 7), and was never replaced by a vasodilator response (Fig. 8) In contrast, after α blocking NA infusion increased ECBF by an average of 20 % (SE 5%) indicating β-adrenergic vasodilator response in the nondental tissues supplied by the external carotid artery

Close intra-arterial infusion of varying concentrations of NA was made for up to 16 min duration. Within this time interval there was no "escape of the vasoconstrictor effect in the dental pulp, i.e. PBF remained low as long as the NA infusion was continued

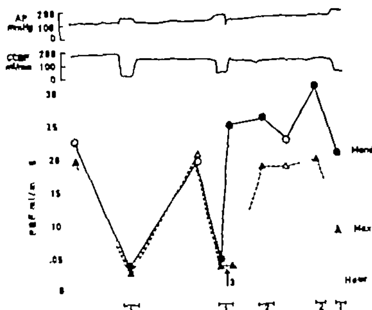


Fig. 6. Simultaneous recordings of systemic arterial pressure (AP), common carotid blood flow (CCBF) and pulpal blood flow (PBF) in ipsilateral upper (triangles—broken lines) and lower (circles—solid lines), canine tooth. Open symbols represent the controls. 1. Cervical sympathetic stimulation 5 V 1 ms, 6 Hz. 2. Inferior alveolar nerve stimulation 5 V 1 ms, 6 Hz. 3. Section of the inferior alveolar nerve in the canal.

Usually the vasoconstriction lasted 1 to 4 min after NA infusion was stopped. Systemic arterial pressure rose slightly in some experiments. Mean pressure averaged 128 mmHg (SE 4 mmHg) in control periods and 132 mmHg (SE 3 mmHg) during catecholamine infusion. The vasoconstrictor response in the dental pulp was clearly dose dependent (Fig. 8). Doses of 5 μg/min completely stopped H_2-washout, while rates lower than 0.01 μg/min gave no measurable change in PBF

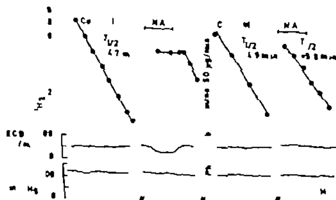


Fig. 7. Effect of close i.a. infusion of noradrenaline (1 μg/min) before and after i.a. infusion of phenoxybenzamine (30 μg/min 15 min) on hydrogen desaturation curves from one electrode in the dental pulp, I_H: Hydrogen oxidation current (semilogarithmic plot). Simultaneous recordings of external carotid blood flow (ECBF) and systemic arterial pressure (AP).

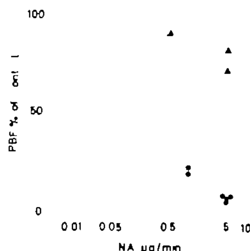


Fig. 8. Effect of various doses (0.01 to 5 μg/ml) of close i.a. infusion of noradrenaline on pulpal blood flow (PBF). Closed circles, before and closed triangles after phenoxybenzamine 7 mg/kg b.w.

Discussion

The experiments demonstrates clearly that the inferior alveolar nerve in the mandibular canal contains sympathetic α-adrenergic vasoconstrictor fibers (Fig. 6). In spite thereof efferent stimulation of the cut inferior alveolar nerve produced variable pulpal flow changes,—even pronounced vasodilation. Moreover, this vasodilation could be reinforced by intravenous infusion of atropine (Fig. 4). Thus, both increase and decrease in PBF were recorded during electrical stimulation of the inferior alveolar nerve, indicating that blood flow in the dental pulp in dogs may be influenced by both vasodilator and vasoconstrictor fibers.

Basal vasoactive nerve tone

Cutting of the cervical sympathetic nerve caused no change in PBF, suggesting nervous sympathetic basal tone in the pulp to be absent or low under the present experimental conditions. This conclusion agrees with the results reported by Edwall (1971), but is difficult to reconcile with the findings that section of the inferior alveolar nerve in the mandibular canal caused vasodilation (Fig. 1). Two possible mechanisms should be considered.

1. The inferior alveolar nerve contains tonically active vasoconstrictor fibers that are not severed by cutting the cervical sympathetic. Since α-adrenergic blockade did not enhance the dilatory effect of inferior alveolar nerve stimulation, this possible vasoconstrictor tone should not be mediated through α receptors.

2. Alternatively, the flow increase induced by inferior alveolar nerve section could be due to mechanical antidromic stimulation of trigeminal sensory fibers. The similar magnitude of the vasodilation caused by inferior alveolar nerve section and that caused by mandibular antidromic nerve stimulation would be compatible with this hypothesis. However, it should be emphazied that the results do not indicate a nervous vasodilator tone during normal conditions.

The possibility that removal of nervous vasoconstrictor tone could be compensated by autoregulation of pulpal blood flow has been excluded by previous investigations (Tonder 1975).

Cervical sympathetic nerve stimulation and NA infusion

Electrical stimulation of the cervical sympathetic and NA infusion consistently increased pulpal vascular resistance. The constrictor effect was greatly reduced by α-blockers, but was never observed to be reversed to a vasodilator effect. Thus, the pulpal resistance vessels seem to be poorly equipped with β-receptors, supporting earlier observations where infusion of isoprenaline gave a weak if any-pulpal vasodilation (Tønder 1976). Atropine, which should block a possible cholinergic vasodilation, had no effect on PBF during electrical stimulation of the cervical sympathetic. It seems therefore justified to conclude that the cervical sympathetic fibers have only one haemodynamic effect on the pulp, namely vasoconstriction, through activation of α-receptors.

Inferior alveolar nerve stimulation

The vasoconstriction elicited by cervical sympathetic stimulation was obviated by cutting the inferior alveolar nerve (Fig. 6), showing that the cervical sympathetic α-adrenergic fibers are contained in the inferior alveolar nerve. However contrary to expectation, stimulation of the inferior alveolar nerve did not consistently cause vasoconstriction (Fig. 2, left panel), and in several experiments a marked flow increase was observed. A similar variability in response was noticed by Weatherred (1965), who reported both an increase and decrease in pulp pressure during inferior alveolar nerve stimulation. The variable flow response could theoretically be caused by varying activation of and β-receptors by NA released at the sympathetic nerve endings. However this hypothesis is unlikely since 1 both responses were obtained with similar stimulation characteristics, and since 2. α-blockade (Fig. 5) and intra-arterial infusion of isoprenaline (Tønder 1976), gave little evidence for β-receptors. The inevitable conclusion therefore seems to be that the inferior alveolar nerve in addition to adrenergic fibers contains fibers which cause pulpal dilatation when activated. As will be discussed later the vasodilator response is most readily explained as an antidromic activation of sensory trigeminal fibers.

However this simple scheme fails to explain two rather surprising observations. In contrast to that observed during cervical sympathetic stimulation,—the constrictor response to inferior alveolar nerve stimulation was 1 reduced by atropine, but was 2. not appreciably influenced by phenoxybenzamine. The effect of atropine suggests that acetylcholine release plays a role in the constrictor response to inferior alveolar nerve stimulation. A constrictor effect of acetylcholine has previously been observed during intra-arterial infusion (Tønder 1976). The vasoconstriction might be due to a direct activation of vascular smooth muscle (Åström et al. 1964), or to enhanced release of catecholamines at sympathetic nerve endings (Ferry 1966). The latter mechanism seems unlikely since phenoxybenzamine had no effect on the response to inferior alveolar nerve stimulation. In fact, the lacking effect of atropine on the response to stimulation of the cervical sympathetic indicates that the cholinergic mechanism is not associated with sympathetic adrenergic fibers.

An effect of atropine on pulpal circulation has previously been reported by Weiss et al. (1972). They observed that electrical stimulation of the cut cervical sympathetic produced a decrease in "intrapulpal blood pressure" in the maxillary canine. This

response persisted in adrenergically depleted or α-adrenergic blocked dogs, but could be reduced or abolished by intravenous administration of atropine. Obviously these results are not in complete agreement with the present findings, but do suggest the presence of a cholinergic vasoactive mechanism in the dental pulp.

Mandibular nerve stimulation

Efferent stimulation of the mandibular nerve before its entry into the mandibular canal caused pulpal vasodilation (Fig. 2, right panel), which seems to indicate release of some vasodilatory substance at the sensory nerve endings. An axon reflex, like that involved in the trippel response in skin (Lewis 1927), would therefore seem possible also in the pulp, and might be of functional importance in the development of inflammatory vasodilation in the infected pulp. In fact the high control PBF measured after exposure of the mandibular nerve (Fig. 2, right panel), might suggest that the surgery itself had caused mechanical antidrome trigeminal stimulation and vasodilation. The prolonged vasodilator action of the unknown transmitter released in a sensory axon-reflex might thus explain why the increase in flow during mandibular nerve stimulation was so modest. We realize, however, that the present experiments are inadequate for a definite characterization of the vasodilator mechanism.

As described in an earlier study (Tønder 1976), vasodilation in neighbouring tissues may cause "stealing" of dental perfusion pressure, thus causing a reduction in PBF. During electrical nerve stimulation, not only the vascular resistance of the dental pulp was influenced, but also resistance in associated tissues. The prerequisite for this "steal" phenomenon is that there normally exists a considerable pressure gradient from the central arteries to the arterial ramification feeding the dental pulp. A pressure drop of on average 11 mmHg has previously been measured from the aorta to the lateral nasal artery (Tønder 1976), and the pressure drop to the small arterioles feeding the dental pulp may probably be of a greater magnitude. In the present study, pressure in the arterioles directly feeding the dental pulp was not measured. However, a simultaneous drop in pulpal perfusion pressure would mask a fall in pulpal vascular resistance during nerve stimulation. Accordingly, pulpal vasodilation may have been underestimated.

To what extent blood flow changes in neighbouring tissues will affect PBF depends upon the normally existing ratio between intrapulpal to extrapulpal vascular resistance in the seriecoupled vascular bed. Further research is therefore necessary to determine the normally existing pressure drop in the dental pulp, i.e. knowledge of the pressure in the small arterioles directly feeding, and the venules draining the dental pulp.

To summarize, both increase and decrease in PBF was recorded during electrical nerve stimulation. Cervical sympathetic stimulation consistently caused a marked fall in PBF due to activation of α-receptors, while mandibular nerve stimulation before its entrance in the mandibular canal resulted in increased PBF in ipsilateral lower canine, probably due to the axonreflex mechanism. Efferent stimulation of the exposed inferior alveolar nerve in the mandibular canal gave variable PBF responses, presumably on account of that the inferior alveolar nerve is a mixed nerve, composed of both sensory, adrenergic sympathetic and cholinergic fibers.

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Frequency selectivity of the peripheral auditory analyzer studied using broad band noise

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Received 16 January 1978

Abstract

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The sharpness of the frequency tuning of single auditory nerve fibers was studied in the rat on the basis of responses to broad band noise The cross-spectra between the sound stimulus and the sound-to-stimulus locked discharge rat were used as estimates of the transfer functions of the peripheral auditory analyzer The sharpness of the tuning obtained in that way was measured as Q_{3dB} and Q_{10dB} It was found that these Q-values decreased with increasing stimulus intensity but that the decrease was different when it was based on measurements at 3 dB points of the obtained transfer functions compared with measuring it at 10 dB points. The change in width was less for fibers with low CF In all the fibers studied the frequency of maximal response (CF) decreased with increasing sound intensity The implication of these findings for pitch perception and for noise induced hearing loss is discussed.

Recent studies have provided much data on the frequency selectivity of the auditory periphery A variety of different methods have been used to assess the frequency selectivity that can be measured in the cochlea, the basilar membrane and in single fibers of the auditory nerve (for reviews, see Møller 1972 and Evans 1975)

During the last two decades, measurements of the linear up-and-down motion of the basilar membrane have been performed in anesthetized animals by several investigators using different methods. The results of these measurements have been contradictory in many respects. Rhode (1971 1973) Robles *et al* (1976) using the Mössbauer effect, find that the motion of the basilar membrane is nonlinear within the physiological range of sound intensities. No other investigators have reported such findings. Johnstone and Boyle (1967) and Johnstone *et al.* (1969), using the same technique but in another animal species, find an essentially linear relationship between sound pressure and amplitude of motion. The results of other studies, in which a capacitive probe has served to measure the basilar membrane motion, also indicate that the basilar membrane function is a linear filter (Wilson and Johnstone 1972).

Several methods have been used to measure the frequency selectivity of the auditory

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periphery at the level of the auditory nerve. The most common method concerns the recording of frequency threshold curves (FTC) of single auditory nerve fibers in response to pure tones (see *e.g.* Kiang *et al.* 1965 and Evans 1975). Iso-intensity curves showing the discharge rate as a function of frequency for constant sound pressure have been determined (see e.g. Rose *et al.* 1971). A few studies have used isorate curves to show the sound pressure necessary to evoke a certain discharge rate (Geisler *et al.* 1974). The results of these studies are difficult to compare because of the influence of the properties of the neural excitation process itself.

Determination of the response of single auditory nerve fibers to a short acoustic impulse (click) also gives information about the frequency selectivity properties of a frequency selective system. Such responses, however are also influenced by the refractory properties of the neurons and do not reflect the frequency selective properties of the system only (see Kiang *et al.* 1965 Gray 1966). Recently stochastic signals have come into use in combination with recordings from single auditory nerve fibers and with statistical signal analysis for determining the filter characteristics of the auditory periphery (de Boer 1968 1969 Møller 1977 a, b, 1978, Evans 1977). This method makes it possible to estimate the frequency selective properties of the auditory periphery on the basis of the discharge pattern in single auditory nerve fibers over a large range of sound intensities (Møller 1977 a, b, Evans 1977). Because the stimulus is a continuous sound the influences of refractoriness on the response pattern is small compared with results obtained in response to stimulation with click sounds. In response to click stimulation the neuron will be refractory for a period following the first time it fires. That period of refractoriness may extend beyond the next excitatory phase of the basilar membrane motion. Using continuous stimulation diminishes these effects of refractoriness and consequently it is assumed that the present method makes it possible to estimate the frequency selective properties of the auditory periphery with a minimum of influence of the refractory properties (Møller 1975).

In previous work (Møller 1977 a, b, 1978), it was shown that the tuning of single auditory fibers in response to broad band noise changes in a systematic way such that the bandwidth of the estimated transfer functions increases with increasing sound intensity. In the present study the variation of bandwidth and center frequency as a function of sound intensity was studied for fibers with different center frequencies.

Methods

White rats weighing 200–300 g ere used. A hole as made in the occipital part of the skull and parts of the cerebellum were sucked away. Recordings were made with hyperfine glass pipettes filled with 2.5 mol potassium acetate and with resistance of 50–100 Mohms. The electrodes were placed in contact with the surface of the dorsal cochlear nucleus and aimed at the auditory nerve' entrance.

The electrode as advanced in 2 µm steps by Ståhlex micromanipulator. The recorded potentials ere amplified by capacitance compensated amplifier (Fonema). During the recording the animal was in sound isolated box. The sound stimuli consisted of pseudorandom noise, generated by Hewlett Packard noise generator (type 3722A). The sound was fed into condenser microphone (Brüel & Kjær type 4131), hich then served as the sound source, via conventional amplifiers and attenuators. The condenser microphone was connected to one of the hollow ear bars of the head holder. For further details about the recordings and sound generation, see Møller (1969, 1971). The sound generating system produced sound

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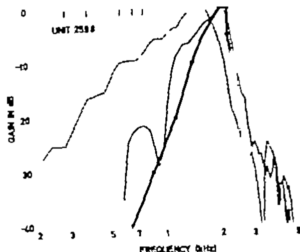


Fig. 2. Cross-spectra (transfer functions) obtained using noise stimuli 15 dB above threshold (thin continuous line) compared with the frequency threshold curve obtained using tone bursts (heavy solid line and dots). The dashed line — the transfer function obtained in response to noise stimuli at an intensity of 70 dB above threshold.

order to be classified as primary units, the units should not show injury potentials, contact with the units should be lost when the electrode was advanced a few microns and the shape of the recorded potentials should be monophasic.

Near threshold, the transfer function in response to noise stimuli of many of the units resembles the frequency threshold curves (FTC) obtained with conventional methods employing tone bursts. Fig. 2 shows a comparison between the frequency curve (heavy line) and the transfer function computed on the basis of noise stimulation at a level near threshold (thin solid line). It is seen that there is a fairly good agreement between the frequency threshold curve (FTC) and the transfer function computed for noise whose level is just above threshold. The dashed line shows the transfer function computed in the same way but for an intensity of 55 dB above that shown by the thin solid line. The transfer function obtained at a 55 dB higher noise level (dashed line) has a broader peak and the curve is shifted downwards in frequency. This widening of the peak and shift downward in frequency are typical findings.

The change in center frequency (CF) and in width of the transfer functions for two typical fibers is further illustrated in Fig. 3 A and B. The peak value is normalized and the various curves shifted 5 dB vertically in order to facilitate comparison. These two units have different CF's and it is seen that the CF shifts downwards as the intensity is increased. The shift in CF is, however, less for the unit with low CF than for the one with a higher CF.

The Q-value (CF values divided by the width of the obtained transfer functions) was higher for fibers with high CF than for those with low. At low sound levels the Q-value increases more rapidly with CF than at high sound levels. This is illustrated in Fig. 4 where the Q_{3dB} and Q_{10dB} (CF divided by the width of the transfer functions measured at 3 resp. 10 dB below the peak value) are shown for the fibers studied. These two figures show individual data points for the individual fibers at the different sound intensities plotted as a function of the fiber's CF with the linear regression lines superimposed. The sound intensities are indicated by legend numbers. The spread of the data is considerable and the linear regression lines may not adequately describe the experimental data.

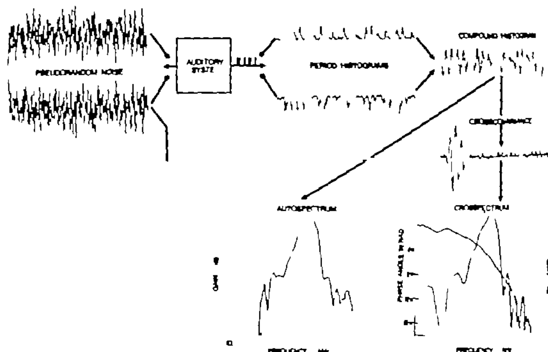


Fig. 1 Schematic illustration of the data processing.

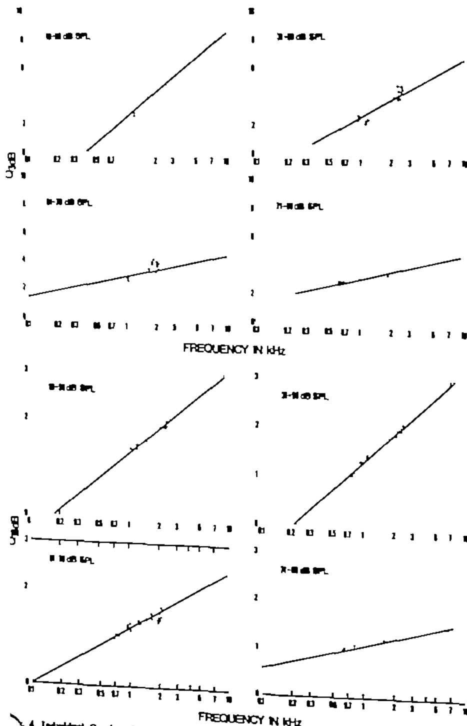
pressure near the eardrum which measured by a probe microphone, varied less than ± 5 dB between 500 Hz and 6 kHz.

The noise generator was set at a clock period of either 10 or 3.33 μs and a sequence length of 4 095 points i.e. each noise period had a duration of 4 095 and 1 364 msec respectively. The noise spectrum was low-pass filtered with an upper cut off frequency of 5 and 15 kHz respectively (Møller 1974, 1977 a). Period histograms of the recorded potentials were made on-line by an Intertechnique DIDAC 800 analyzer. The analyzer was synchronized with the periodicity of the noise. The binwidth of the period histogram was 20 μs when 15 kHz noise was used and 60 μs when 5 kHz noise was used. The polarity of the noise was reversed during half the time of each recording and summation of discharges was changed to subtraction (see Fig. 1).

The compound histograms were processed off line on a general purpose computer to produce cross-covariance functions. Further computation then yielded auto- cross- and coherence spectra (Møller 1974, 1977 a). The histograms contained 682 bins while the auto- and cross-spectra were being obtained using 1 024 data points. The cross-covariance functions were regarded to be valid approximations of the impulse response of the system under test and the Fourier transforms of these cross-covariance functions (cross-spectra) were regarded to be valid approximations of the transfer function from sound to modulation of discharge rate (density) of these primary fibers. Although the cross-covariance analysis used to process these histograms is insensitive to nonlinear distortion such as half wave rectification, there are certain advantages in using compound histograms as basis for computation of the cross-covariance. For example, the higher even-ordered distortion components originating from the special properties of the noise used are eliminated by this procedure.

Results

The present study is based on recordings from 64 fibers. Units were classified as primary fibers on the basis of the same criteria as used in previous studies (Møller 1976, 1977 a) with one exception, namely units with a characteristic frequency (CF) below 1 200 Hz were classified as primary fibers if their latency was below 3 ms. Among the units with CF above 1 200 Hz only those with latency below 2 ms were included in the study. In addition, in



4 Individual Q-values for the fiber studied plotted as function of CF. Each data point is the
intensity ranges indicated in each of the four graphs. The straight lines are linear

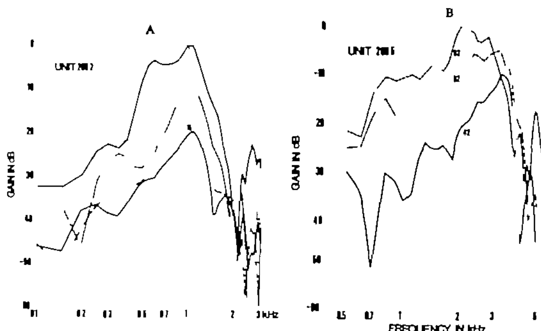


Fig. 3 Frequency transfer functions obtained in two different fibers. The individual curves were obtained at different sound levels, indicated by legend numbers, in dB SPL measured in a ⅓ octave band. The curves are shifted vertically in such a way that there is a 5 dB difference between the maximal values of the individual curves. This was done in order to facilitate comparison of the width and center frequency of the individual curves.

The change in CF as a function of sound intensity is shown in Fig. 5 A for units with CF below 1 400 Hz and in Fig. 5 B for units with CF above 1 400 Hz. It is seen that the CF of all the units decreases with increasing sound level, but that the spread between units is much greater for the units with CF below 1 400 Hz than for those with CF above 1 400. For units with CF above 1 400 Hz a systematic and gradual decrease in CF with intensity is seen. The decrease in CF is most pronounced for intensities above 40 dB SPL. At 80 dB SPL the CF has decreased to between 70 and 80% of the value it had at intensities near threshold. The data given in Fig. 5 concern units on which results were obtained over a large range of intensities and thus does not represent the total material.

Discussion

In the present study the change in the width and CF of the computed transfer function (cross-spectra) of primary auditory fibers in response to broad band noise as a function of the intensity of the noise was studied. The change in the width of the transfer functions of primary fibers was found to reveal a different course depending on whether the width of the derived transfer functions was measured at 10 dB points or 3 dB points. The CF decreases with increasing intensity in all the units studied.

Frequency threshold curves (FTC) measured in the cat by Kiang *et al* (1965) showed higher Q_{10dB} values than those obtained in the present study at noise levels just above threshold. Kiang's data showed a mean Q_{10dB} value of about 3 for fibers with CF at 1 kHz compared with 1.3 in the present study for intensities near threshold. At 3 kHz, the

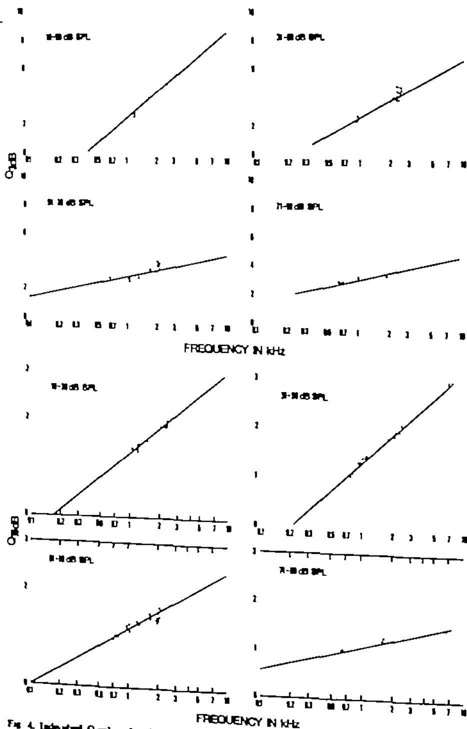


Fig. 4. Individual Q-values for the fiber studied plotted as a function of CF. Each data point is the Q-value obtained in the intensity ranges indicated in each of the four graphs. The straight lines are linear regression lines.

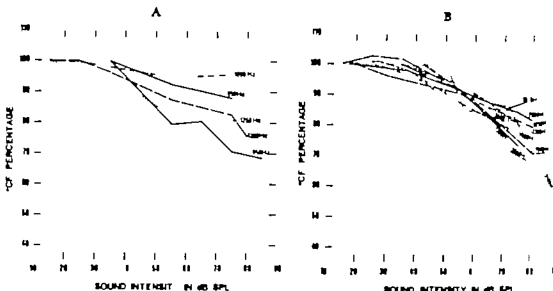


Fig. 5 Change in CF as a function of sound level (given in dB SPL in ⅓ octave band) for a number of typical units. The ordinate is CF in percentage of its value at intensities near threshold. The CF of the units is given by legend numbers. A Units with CF below 1 400 Hz, B Units with CF above 1 400 Hz (736)

Q_{10dB} for the FTC's obtained in the cat is about 6 whereas the Q_{10dB} obtained in the present study at 10–30 dB SPL is about 2.3 At 4 kHz, the mean value of the Q_{10dB} of the FTC's is greater than 10 The Q_{10dB} determined on the basis of the responses to noise stimulation was about 2.5 near threshold for fibers with CF around 4 kHz. The above given Q_{10dB} of FTC in the cat (Kiang *et al* 1965) agrees well with the Q_{10dB} in the guinea pig reported by Evans and Wilson (1973)

It is thus obvious from these results that the FTC's obtained using pure tones are narrower (at 10 dB points) than the transfer functions obtained in the present study using noise stimuli Despite this general rule, the FTC and transfer functions in response to noise in an *individual* fiber may resemble each other (*cf* Fig. 2) as has been shown by de Boer (1969) as well.

It is also of interest to compare the frequency tuning of the basilar membrane shown by Rhode (1971 1973) in the squirrel monkey with the results of the present study He found a tuning at 8 kHz and 70 dB SPL that had a Q_{10dB} of about 4 The present study does not include fibers with CF as high as 8 kHz but extrapolation suggests that Q-values for such CF's would be less than 4 at 70 dB SPL.

It is seen from Fig. 3 that the change in bandwidth with increasing sound intensity is mainly a result of a decrease of the low frequency slope of the transfer functions. The high frequency slope is almost unchanged. The slope of the low frequency part of the transfer functions is to a great extent a result of the transmission characteristics of the middle ear The *absolute* value of the bandwidth consequently becomes greatly dependent on transmission properties of the middle ear The characteristics of the middle ear however do not change with sound intensity Thus, although the bandwidth measurement may not be a good measure of frequency selectivity the *change* in bandwidth with sound intensity may represent a true measure of the change in the frequency selectivity of the peripheral analyzer with sound intensity

The shift in CF downwards in frequency with increasing sound intensity seen in the present study implies that the maximal deflection of the basilar membrane, when produced by a pure tone, shifts towards the base of the cochlea. Indeed, psychoacoustic experiments show no dependence of pitch perception on intensity (Terhardt 1975) (except for pure tones where there is a slight change in pitch with intensity). The change in CF observed in the present study on the other hand may explain why noise induced hearing loss caused by noise in the form of pure tones or narrow band noise as well as the temporary threshold shift after exposure to such sounds is greatest at a frequency about one half to one octave above the frequency of exposure. A hearing-damaging sound with a narrow band (as *e.g.* a pure tone or narrow band noise) will hence damage the hair cells at a point of the basilar membrane which, at threshold intensities, responds to a frequency that is higher than the frequency of the sound in question.

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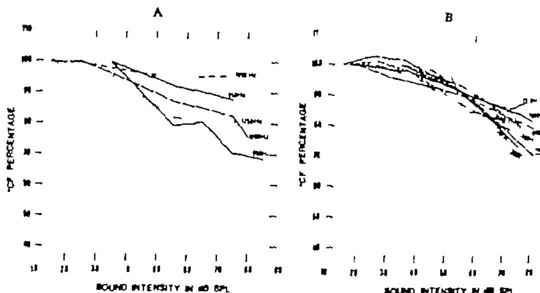


Fig. 5. Change in CF as a function of sound level (given in dB SPL in ⅓ octave band) for a numbe typical units. The ordinate is CF in percentage of its value at intensities near threshold. The CF of units is given by legend numbers. A Units with CF below 1 400 Hz, B Units with CF above 1 400 (736)

Q_{10dB} for the FTC's obtained in the cat is about 6 whereas the Q_{10dB} obtained in the prese study at 10–30 dB SPL is about 2.3 At 4 kHz, the mean value of the Q_{10dB} of the FT(is greater than 10. The Q_{10dB} determined on the basis of the responses to noise stimulati was about 2.5 near threshold for fibers with CF around 4 kHz. The above given Q_{10dB} FTC in the cat (Kiang *et al.* 1965) agrees well with the Q_{10dB} in the guinea pig reported Evans and Wilson (1973)

It is thus obvious from these results that the FTC's obtained using pure tones narrower (at 10 dB points) than the transfer functions obtained in the present study usi noise stimuli. Despite this general rule, the FTC and transfer functions in response noise in an *individual* fiber may resemble each other (*cf* Fig. 2) as has been shown by Boer (1969) as well

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From the Institute of Neurobiology and Dept. of Surgery III, Sahlgren's Hospital, University of Göteborg, Sweden

Efferent innervation of the small intestine by adrenergic neurons from the cervical sympathetic and stellate ganglia, studied by retrograde transport of peroxidase

By

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Abstract

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The nervous pathways between the small intestine of cat and guinea pig and various sympathetic ganglia were investigated by the retrograde horse-radish peroxidase (HRP) technique. HRP was injected at multiple sites in the wall of the duodenum and the first third of the jejunum. At 1–5 days after the injections, the HRP reaction product was searched for in various sympathetic ganglia. Not only the coeliac and nodose ganglia, but also the superior cervical, medial cervical, stellate and thoracic ganglia contained HRP positive nerve cells. Crushing the cervical vagal nerve prevented the occurrence of HRP-reaction in the cervical ganglia, indicating that the HRP was transported from the gut to the cervical ganglia via axons in the vagal nerve. The results demonstrate that the sympathetic ganglia in the neck (sup. and med. cerv. ganglia and stellate ggl.) send efferent fibres to the small intestine.

Key words: mammalian duodenum, HRP injection, retrograde transport, adrenergic perikarya, cervical sympathetic ganglia, vagus nerve

Several observations indicate that the serotonin (5-HT) content in enterochromaffin cells in the small intestine of mammals may be influenced by stimulation of efferent nerve fibres in the cervical vagus (Hohenleitner *et al.* 1971, Tansy *et al.* 1971, Ahlman *et al.* 1976 a). If adrenergic β-receptor-blocking agents were administered prior to nerve stimulation, or if the superior cervical ganglia (SCG) were removed 1 week before the stimulation experiment, the release of 5-HT from enterochromaffin cells was abolished (Ahlman *et al.* 1976 b). This indicated that an adrenergic pathway, running in the cervical vagus, was causing the 5-HT release by liberating a catecholamine (CA). By nerve crush experiments it has been shown that the cervical vagal nerve contains descending CA-containing fibres, probably originating in the SCG (Muryobayashi *et al.* 1968, Liedberg

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TABLE I The presence of HRP reaction product in neuronal structures of the guinea pig after loc. injections of HRP in the wall of the small intestine HRP-injections and nerve crush operation performed 24 h before death. + Presence of HRP-positive cells or fibres 0 = no HRP product observed – = not studied.

Structure	Crush operation			
	– (n = 3)	cerv vagus unilat. (n = 2)	cerv vagus + cerv preggl. nerve unilat. (n = 2)	cerv vagus bilat. (n 2)
Cerv vagus				
ipsilat	–	+	+	+
contralat.	–	–	–	+
Cerv preggl. nerve:				
ipsilat.	–	0	0	–
contralat.	–	–	–	–
Coeliac ggl	+	+	+	+
Stellate ggl				
ipsilat.	+	+	+	+
contralat.	+	+	+	+
Sup. cerv ggl				
ipsilat.	+	0	0	0
contralat.	+	+	+	0

et al 1973 Ahlman *et al* 1976 b) However the direct connection between these fibres and the small intestine was never established. The purpose of the present investigation was therefore to perform experiments which could demonstrate a direct morphological pathway between the small intestine and the SCG

Exogenous proteins are known to be taken up by nerve terminals of various kinds of neurons and transported *via* retrograde intra-axonal transport towards the cell body A protein which has been frequently used for studies of retrograde intra-axonal transport and for tracing nervous pathways is horse-radish peroxidase (HRP) (*cf* Kristensson and Olsson 1971 Ellison and Clark 1975). This protein was therefore used in our studies and injected into the small intestine and traced, by histochemical procedures, in various ganglia and the vagal nerve.

Material and methods

Injections of HRP 9 adult male guinea pigs and 7 adult cats of both sexes were used. HRP (Type II Sigma) dissolved in saline (> 50 %), was injected at multiple sites into the wall of the duodenum and the proximal ½ of the jejunum. The injections were made under aseptic conditions t laparatomy under Nembuthal (cats, 35 mg/kg i.p.) or ether (guinea pigs) anesthesia. The total volume of HRP-solution injected into each nimal was 250 (guinea pig) or 500 (cat) µl, and each injection consisted of 1 3 µl of the solution (freshly prepared for each animal). The survival time was between 1 5 days (see Table I nd II). The postoperative care of the cats consisted of heating pads or lamps during the recovery after anaesthesia, and fluid administration if necessary (indicated by weight loss) during the following days. W ter and food was supplied *ad libitum*.

Nerve operations: In 6 of the 9 guinea pigs the cervical vagal nerve alone or together with the adjacent cervical sympathetic preganglionic nerve was crushed, uni- or bilaterally (T ble I). In 3 cats the right cervical vagal nerve, together with the adjacent cervical preganglionic nerve were crushed 0 or 24 h (Table II) after the HRP injections. The crushes were performed a.m. Lubińska (1959) with silk suture

TABLE II. The presence of HRP-reaction product in neuronal structures of the CAT after local injections of HRP in the wall of the small intestine. Nerve operations performed as indicated. + = HRP positive structures present, 0 = no HRP reaction product observed, — = not investigated.

Cat No.	I	II	III	IV	V	VI	VII
Time inj. – unilat. nerve op.	0 h	24 h	24 h	—	—	0 h	—
Time nerve op. – death	24 h	36 h	48 h	—	—	3 d (subdiaph. vag. cut bilat.)	—
Total survival time:	24 h	60 h	3 days	2 days	3 days	3 days	5 days
Structures							
Cerv. vagus:							
ipsilat.	0	+	+				
contralat.		—	—				
Cerv. pregg. nerve:							
ipsilat.	0	0	0				
contralat.	—	—	—				
Coeliac gl.	+	+	+	+	+	+	+
Stellate gl.							
ipsilat.	0	+	+	+	+	0	+
contralat.	0	+	+	+	+	0	+
Med. cerv. gl.							
ipsilat.	—	+	+	—	—	—	—
contralat.	—	+	+	—	—	—	—
Sup. cerv. gl.							
ipsilat.	0	0	0	0	+	0	+
contralat.	0	+	+	0	+	0	+
Nodose gl.							
ipsilat.	0	0	0	0	+	0	+
contralat.	0	+	+	0	+	0	+
Various thoracic gl:		—	—	—	—	—	+

(3 0) under the nerve(s) which was pulled firmly (3–5 s) against the glass rod placed over and along the nerve(s). This produced a straight and very clear interruption of the nerve(s) but with intact connective tissue sheaths. In one cat (Table II) both subdiaphragmatical nerves were traced and cut at the time of the HRP injection.

The histochemical demonstration of HRP. At the end of the survival period, guinea pigs were reanaesthetised with ether and cats with Nembutal. Following a brief pre-perfusion (30 s) with Ringer-Rheomacrodex (Pharmacia) the animals were then perfusion-fixed with ice-cold 2.5% glutaraldehyde in 0.15 M phosphate or Tris-buffer, pH 7.4. After dissection of appropriate tissues these were postfixed for 2 h (guinea pigs) or 5 h (cats) in the same fixative at +4°C. The specimens were rinsed in buffer with 5% sucrose over night and then sectioned at about 30 μm (ganglia) or 10 μm (nerves) in a cryostat at −18–−20°C. The sections were then incubated with 3, 3'-diamino benzidine (DAB) followed by 0.01% hydrogen peroxide according to Graham and Karnovsky (1966) or with the modifications described by Malmgren and Olsson (1977). The sections were then mounted in 50% glycerine and stored at −20°C until examined in a phase contrast microscope. The DAB-incubation procedure caused a dark brownish precipitate over areas with HRP-activity. Granules with HRP-activity were in most cases clearly different from the yellowish lipofuscin pigment present in many cells. In doubtful cases, however, the reaction was studied in a fluorescence microscope, in which lipofuscin granules are strongly yellowish autofluorescent while the HRP-reaction product is dark. Kodak Tri-X film was used for photographs.

Dissected tissues were: The coeliac ganglion (mesenteric inferior ganglion), the stellate, medial cervical (MCG), superior cervical (SCG) and nodose ganglia bilaterally and the thoracic chain ganglia (cat 7 Table II). When nerve crush operations were performed the cranial end of the nerves caudal to the crush [illegible] dissected out.

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contralat.	+	+	+	+
Sup. cerv ggl				
ipsilat.	+	0	0	0
contralat.	+	+	+	0

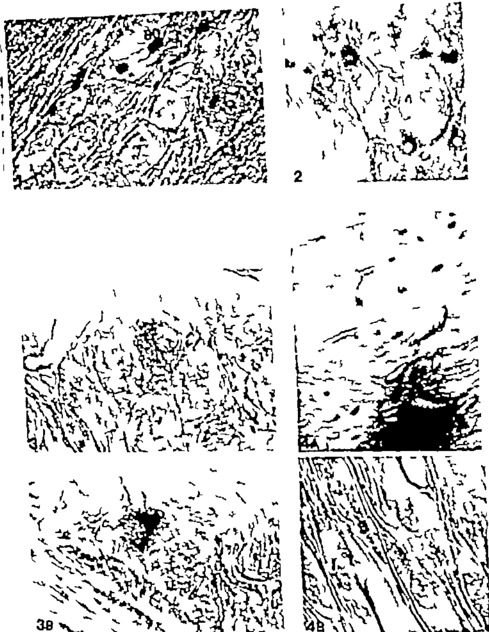
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Figs. 1–4

caudal to the crush (Fig. 8). In the cervical sympathetic trunk no accumulation of HRP product was demonstrated in relation to the crush (see Table I and II). These experiments also show that in cat the retrograde transport of HRP had not reached the level of the crush at 24 h.

Results

In both species the injection of HRP into the small intestine caused dark reaction product to appear after the DAP-procedure in cell bodies of all ganglia investigated after a sufficient transport time, *i.e.* celiac, stellate, nodose ganglia, MCG and SCG (Table I and II). There was a positive reaction in cell bodies of all these ganglia in guinea pigs already 4 h after injection (Fig. 1). In cats only the celiac ggl had HRP positive cells 24 h after injection (Fig. 2). If the observation time of cats was 60 h (cat II Table II), relatively few cell bodies of the cervical ganglia contralateral to the nerve crushes contained HRP-reaction product, and the amount of product in the positive cells was rather small (Fig. 3). After 72 h, however cats III V VII (Table II), the number of positive cells had increased, and more material seemed to have accumulated in the cells (Fig. 4 5). In the cervical sympathetic ganglia of the 3 days surviving cats the HRP reaction product was distributed fairly evenly in the few positive cells observed. In the 5 days cat, however (cat VII Table II), the HRP reaction product was often accumulated near one or both poles of the nucleus, thus resembling the location of lipofuscin pigment in these neurons (Figs. 4 5).

By studying these cells in the fluorescence microscope it was demonstrated, however that these granules were not yellowish autofluorescent as is lipofuscin. They rather appeared dark in the fluorescence microscope (Fig. 6).

The thoracic ganglia also contained a few HRP positive cells, but the amount of HRP in these cells was usually rather small (Fig. 7).

Crush operation of the cervical vagal nerve uni or bilaterally 0 or 24 h after the HRP-injection prevented in both guinea pigs and cats the occurrence of HRP reaction product in the supraclavicular ganglia of the ipsilateral side thus the nodose ganglia, the SCG and the MCG contained no visible HRP-positive cells. The ganglia on the contralateral side of unilaterally operated animals had positive cells (Table I and II). The infraclavicular ganglia, *i.e.* the stellate and the coeliac ganglia, had the same appearance as in the unoperated animals. *The crush-operated animals were also used as a control that no blood-borne* HRP *was causing the positive results in the supraclavicular ganglia. The vagal nerve of these* crush-operated animals contained many axons with accumulated HRP-reaction product

Fig. 1 Nodose ganglion of n HRP-i jected but not nerve-crushe operated guinea pig, containing one HRP-positive (arrows) and several negative nerve cells. BC indicat red blood cells with endogenous peroxidase activity 270.

Fig. 2 Coeliac ganglio from a cat injected w th HRP 3 days earlier (cat III, Table II). M ny HRP positive cells, with various mounts of HRP-granules n the cytoplasm, can be seen. 70

Fig. 3 A, B. From the stellat ganglion of cat i jected with HRP in the small intestine 60 h (cat II T ble II) before death. Single HRP-positive cells are scatt red amongst many negativ cell bodies. Few (A) or many (B) HRP granules are distributed rather evenly in the cytoplasm. 270.

Fig. 4 Stellate ganglion of a cat i jected w th HRP in the small intestine 5 d. before death. A) Many cell bodies with HRP-reaction product are observed. Not the irregula distribution of HRP to one or two poles of the nucleus. 65 B) Three HRP positive cells with the HRP-granules located ne one pole of the nucleus. These granules were not fluorescent in the fluorescence microscope and are thus not lipofuscin pigment ×270.

Discussion

The HRP-method for studying retrograde transport in axons, and thereby tracing nervous pathways, has been used for some years with excellent results (*cf* Kristensson 1975). In all types of neurons investigated so far including peripheral autonomic neurons, HRP is taken up into the nerve endings and transported by intra-axonal retrograde transport to the perikarya (*cf* Kristensson and Olsson 1971 1973 LaVail and LaVail 1972, Ellison and Clark 1975). The rate of transport towards the cell bodies is rapid, probably exceeding 120 mm/day in the cat and guinea-pig (Ellison and Clark 1975). This figure is in good agreement with our observations that in the cat no HRP-positive cell bodies in the neck ganglia were labelled at 24 h after the HRP-injection, while at 60 h (cat 2 in Table II) some cells were labelled with numerous granules of HRP-reaction product. In the guinea-pig, however strongly positive cells were observed at all levels 24 h after the HRP-injection. The distance between the duodenum and the cervical ganglia was estimated to be at least 30 cm in the cat and about 10 cm in the guinea-pig, indicating a rate of transport of more than 100–120 mm/day for both species.

Autonomic nerves are considered to be particularly suitable for HRP-studies due to their large surface/volume relationship (Nauta *et al.*, 1974). Also, the more terminals belonging to particular perikaryon that are present in the HRP-injected area, the more HRP is taken up and transported to the cell body (Jones 1975). Thus, the amount of HRP-granules present in a cell body can be related to its amount of nerve terminals in the injected area. This may explain why at a certain survival time a particular ganglion may contain cell bodies which are filled with HRP-granules close to other cell bodies with only a few HRP granules or no granules at all. This was observed in all ganglia investigated, but particularly evident in the coeliac ganglion (Fig. 2).

In this study all ganglia investigated after a sufficient transport time contained HRP granules after injection of this exogenous protein into the small intestine. This is unlikely to be due to uptake of blood-borne HRP for two reasons. 1) in animals with nerve crushes

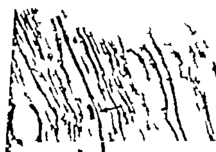
Fig. 5 Cervical superior ganglion of a cat injected [illegible] HRP in the small intestine 5 d. before death (cat VII in Table II). A) T[illegible] cells (arrows) [illegible] rather small amount of HRP-reaction product in the cytoplasm are seen [illegible] 65 B) Two HRP-positive cells [illegible] few HRP-granules, located near one pole of the nuclei can be observed (arrows). These granules were non-fluorescent in the fluorescence microscope and are therefore not lipofuscin granules. 290.

Fig. 6 Set of photographs of [illegible] stellate ganglion of cat VII in Table II. A) Phase contrast picture demonstrating dark HRP-reaction product in one cell (arrow). A few dark red blood cells are also seen (→). B) Fluorescence microscope picture of the same area as in A. The HRP-reaction product in the nerve cell appears dark (arrow), and the same [illegible] the case [illegible] the red blood cells, also containing the DAB-peroxidase reaction product (→). 65.

Fig. 7 Ganglion Th 5 in the thoracic sympathetic chain of [illegible] cat injected with HRP in the small intestine 5 days before death (cat VII in Table II). A) Two HRP-positive nerve cell bodies (arrow heads) and several negative cells (arrows) are observed. 65. B) One nerve cell [illegible] HRP-granules accumulated in the cytoplasm in one pole of the cell. These granules [illegible] are non-fluorescent and thus probably not lipofuscin pigments. 290.

Fig. 8 The distal end of the cervical vagal nerve of cat II (Table II). 24 h after the HRP injections into the small intestine the [illegible] vagal nerve [illegible] crushed (site of crush indicated by arrow head). The animal was killed 36 h. later. Dark brownish granular HRP reaction product can be seen in axonal structures caudal to the crush (arrows). 290.


5A




6B


5B


7A



ganglia, probably via the vagal nerve. The evidence for this is that when the vagal nerve, with or without intact cervical preganglionic trunk, was crushed, HRP was found in accumulated fibres distal to the crush but not in the cervical preganglionic nerve. Crushing of the cervical preganglionic trunk alone could not prevent the appearance of HRP in the SCG (Table I), nor were HRP-accumulations observed in this nerve in either cats or guinea-pigs. It is therefore probable that some of the HRP-containing nerve fibres in the cervical vagus may correspond to the CA-fibres in the vagal nerve which originate in the SCG (These fibres disappear after removal of the SCG). However the vagal nerve fibres with HRP-accumulations may also correspond either to afferent fibres, constituting the major part of the vagal nerve (Agostoni *et al.* 1957), to efferent parasympathetic nerve fibres, or to polypeptide containing nerve fibres in the vagal nerve (Lundberg *et al.* unpublished).

The cervical sympathetic ganglia of cat, and probably also of guinea pig, contain not only adrenergic perikarya but also a few non-fluorescent, acetyl-choline-esterase (AChE)-positive cells which probably are cholinergic (*cf* Hamberger *et al* 1965). These cells are not possible to recognize unless specific AChE-staining is performed. So far no attempt has been made to identify the HRP-positive cells in the present study as adrenergic or cholinergic nerve cells. However 1) cholinergic neurons of the cervical and stellate ganglia of cat are considered to innervate eccrine sweat glands (*cf* Dale and Feldberg 1934 Sjöqvist 1963), and 2) the subdiaphragmatic vagal nerve of cat contains many descending adrenergic fibres emanating from the cervical and stellate ganglia (Liedberg *et al.* 1973 Ahlman *et al* 1976 b). Therefore it is probable that the HRP-positive perikarya are adrenergic in nature rather than cholinergic.

The function of this presumably adrenergic fibre system from the SCG and MCG to the intestine is so far not clear However it may participate in the vagal adrenergic release of 5-HT from enterochromaffine cells, which is caused by selective efferent stimulation of the cervical vagal nerve (Ahlman *et al* 1976 b) or in the vagally induced inhibitory motor responses from the small intestine (Martin *et al.* 1974).

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The *coeliac ganglion* is well known to send postganglionic adrenergic fibres to the stomach and the small intestine (*cf* Furness and Costa 1974) The most likely anatomical pathway for this innervation and for the HRP-transport is the postganglionic fibres which *via* blood vessels reach the gut

It has been suggested earlier that the *stellate ganglion* mainly supplying the heart and cervical-thoracical structures with CA-fibres, also sends CA-fibres to the small intestine (Liedberg *et al* 1973) The number of HRP positive cells in this ganglion was rather great, especially in the 5 days cat. The HRP is likely to have reached the stellate ganglia via the vagal nerve and communicating branches between the stellate and other intrathoracic ganglia and the thoracic portion of the vagus This is supported by the observation that many CA fibres, presumably originating mainly from the stellate ganglia, are present in the vagus at the subdiaphragmatic level after removal of the SCG (Liedberg *et al* 1973 Ahlman *et al* 1976 b; see also Ahlman and Larsson 1978)

The SCG and MCG were classically considered to innervate adrenergically the structure of the head and neck. The present study demonstrates that some cell bodies in this ganglion also supply CA fibres to the small intestine, which is a most surprising observation (Fig. 6) Earlier methods for tracing nervous adrenergic pathways were rather insensitive compared to the HRP-method. Such methods included on the one hand, nerve crush experiments which demonstrated the presence of adrenergic fibres in a peripheral nerve and also gave information on the direction in which the fibres were running On the other hand denervation experiments were performed in which a particular ganglion or set of ganglia was removed about 1 week before fluorescence histochemical or quantitative investigations on a possible decrease in CA nerve terminals in a certain area (*cf* Hamberger and Norberg 1964). With the use of the HRP method, which may demonstrate even a very small relative innervation, it is possible to study the autonomic innervation to the gastro-intestinal tract in much greater detail than earlier

The observations show that HRP is transported from the small intestine to the cervical

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The relationship between cortical recruiting responses and ponto-geniculo-occipital waves during paradoxical sleep in the cat

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Abstract

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In an earlier study it was found that during paradoxical sleep (PS) thalamo-cortical recruiting responses (RRs) and rapid eye movements usually did not appear simultaneously. As shown elsewhere, ponto-geniculo-occipital (PGO) wave activity and rapid eye movements are during PS closely related to each other in time. Similarly in the present study it was observed that during paradoxical sleep in cats in which the center median nucleus of the thalamus was being stimulated at a rate of 7–9 Hz PGO waves in the geniculate nuclei did not as a rule occur in the presence of RRs recorded from the motor cortex. This effect was most pronounced with respect to series of PGO waves which usually occur at a rate of 4–7 Hz. On the basis of these experiments it was concluded that PGO waves and RRs are reciprocal events and mutually exclusive. Considering the well-known fact that RRs represent synchronization, this negative correlation between RRs and PGO waves indicates that the desynchronizing tendency typical to paradoxical sleep is most pronounced during the occurrence of PGO waves.

Key words: Paradoxical sleep, ponto-geniculo-occipital waves, recruiting responses

Since Dempsey's and Morison's original discovery (1942 a, b) concerning the recruiting response (RR) which is seen in the cortex during the repetitive stimulation of the non-specific thalamic nuclei, there have been numerous studies in attempt to clarify this thalamo-cortical relationship and especially its functional significance at various levels of vigilance. Though Dempsey and Morison in their original publication in 1942 studied RRs only in cats under barbiturate anesthesia, it was later shown that RRs could be elicited also during slow wave sleep (S) (Evarts and Magoun 1957, Rossi *et al.* 1961) and—if the thalamic stimulation was slightly suprathreshold compared to that during S—even during certain parts of paradoxical sleep (PS) and quietly alert state (Evarts and Magoun 1957, Yamaguchi *et al.* 1963, Yamaguchi *et al.* 1964). Earlier (Allison 1965, Lehtinen and Valleala 1969) it was shown that RRs and rapid eye movements during PS did not usually occur simul-

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taneously. In the present study it was found that ponto-geniculo-occipital (PGO) waves did not either usually occur in the presence of RRs during PS. A preliminary report on the present study has been published (Laihinen and Valleala 1977).

Methods

The experiments were performed on 4 adult unanesthetized chronic cats carrying teflon-coated stainless steel electrodes previously implanted under barbiturate anesthesia. During the experiments the cats were sleeping on a hammock with the head firmly fixed to a frame by a dental cement pedestal built on the skull. Stimulating electrodes consisted of two wires, each 0.25 mm in diameter. They were insulated to their obliquely cut ends, cemented side by side and their tips were separated vertically by 0.5 mm. The stimulating electrodes were inserted in the left center median nucleus according to Jasper and Ajmone-Marsan's atlas (1954).

The stimuli consisted of rectangular pulses of 0.5 ms duration at a rate of 7–9 Hz. Stimulation was provided by a Grass S-4 stimulator and an RF isolation unit. The stimulus strength needed to elicit RRs during PS was slightly suprathreshold compared to that which had to be used during slow sleep. The recruiting responses were recorded from the ipsilateral anterior sigmoid cortex by using a transcortical electrode set and were identified by their long latency (about 20 ms), surface negative incremental growth and amplitude modulation of waxing and waning character. The PGO waves were detected with bipolar electrodes in the lateral geniculate nuclei.

After experimental series which could last several weeks the positions of the stimulating electrodes and those in the geniculate nuclei were marked by electrolytic deposits of iron. The points were then checked histologically in serial sections which were stained with cresyl violet (Nissl).

Results

In Fig. 1 there is a sample of PS with a recruiting response which was elicited by means of the repetitive stimulation of the center median nucleus and recorded from the anterior sigmoid cortex. At the beginning of the sample the stimulus strength (7 V) is not sufficient to evoke RRs. After a slight increase in the stimulus strength (to 8 V) the recording shows with every pulse a long latency (about 20 ms) potential. At the very beginning of the repetitive stimulation these potentials do not exhibit any systematic amplitude modulation. These potentials have been called sustained waves (Wells and Sutin 1964). However in the middle of the sample, a recruiting-like amplitude modulation can be seen. There is an essential difference between the classical RR and the recruiting phenomenon described in Fig. 1

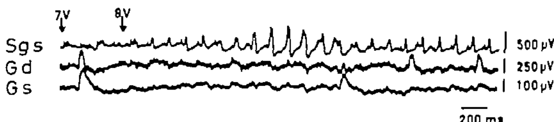
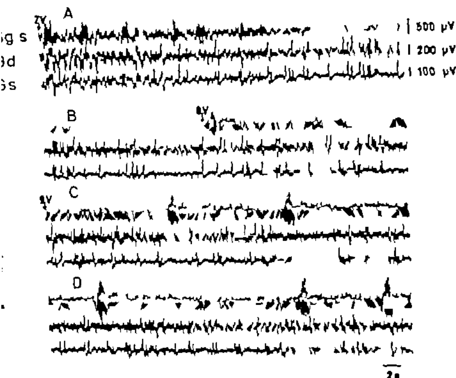


Fig. 1 Electrical activity of the left anterior sigmoid cortex (Sg s) and the right (G.d) and left (G.s) lateral geniculate nuclei. The cortical recording shows recruiting response (RR) elicited by repetitive stimulation (9 Hz, 0.5 ms) of the left center median nucleus. After the increase in the stimulus strength from 7 V to 8 V every stimulus evokes in the left anterior sigmoid cortex long-latency surface negative wave ("sustained wave") which in the course of stimulation shows a recruiting-like amplitude modulation. During RR there are no ponto-geniculo-occipital (PGO) waves in the tracing from the right and left lateral geniculate nuclei. Upward deflection signifies here and in Fig. 2 surface negativity



ig. 2. Continuous recording during the transition from S to PS (tracing A) and PS proper (tracings B–D). halamic stimulation (9 Hz, 0.5 ms) is going on the whole time. The stimulus strength, which was at the reshold level for the occurrence of RRs during S, had to be raised to a higher level in order to evoke current RRs during PS. Sg s., G d. and G s. as in Fig. 1. PGO wave activity of G d. and G s. does not ccur during RRs but may start immediately after the end of each RR (e.g. tracing D)

i.e. that in the classical case the incremental change begins from the zero level whereas in his atypical case during PS the incremental rise is initiated from the level of the sustained waves. There are no PGO waves in the lateral geniculate nuclei during the RR (Fig. 1).

Fig. 2 shows 4 tracings (A–D) representing the same continuous recording during which the repetitive thalamic stimulation is going on the whole time. In the tracing A, the transition from S to PS is shown. It is noteworthy that S changes to PS in spite of the simultaneous thalamic stimulation. In this tracing there are RRs evoked at the threshold stimulation during S. The last few RRs during S overlap the first PGO waves heralding PS, but do not occur strictly simultaneously with these waves. The tracings B–D in Fig. 2 represent the fully developed PS. At the beginning of the tracing B before increasing the stimulus strength, neither sustained waves nor RRs are seen. After an increase in stimulus strength (in the middle of tracing B) there appears an initial RR and sustained wave activity, but not until a further increase in stimulation voltage (at the beginning of tracing C) do the recruiting responses become recurrent. In the presence of RRs PGO waves are absent not only in the left geniculate nucleus but also contralaterally in the respective nucleus.

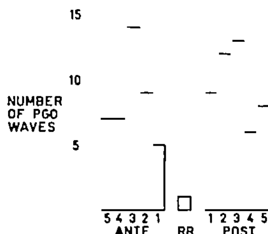


Fig. 3 Number of PGO waves in both the lat geniculate nuclei during a PS episode of 3 length. The repetitive stimulation of the left cen median nucleus was going on the whol PS epis eliciting totally ten RRs, the average length of amounted to 1.3 s. PGO waves were sampled du these RRs and in five 1 s periods before (ANTE) after (POST) RRs. For details, see text

Fig. 3 shows the number of PGO waves in both the lateral geniculate nuclei during a episode of 3 min length The repetitive stimulation of the center median nucleus was goi on the whole PS episode eliciting totally ten RRs, the average length of which amounted 1.3 s. The time immediately preceding and following every RR of the sample was divid into five 1 s periods and the total number of PGO waves occurring in both lateral genicula nuclei was determined separately for each period and during RRs. Fig. 3 shows that on once did a PGO wave occur during RR The typical series of PGO waves at a rate of 4- Hz never occurred during RRs.

Discussion

The results of the present study point out that PGO waves and RRs are aspects of oppos physiological tendencies. Generally PGO waves refer to an increase and RRs to a decrea in the level of activation. The electrocortical desynchronization and the PGO wave activi are among the main bioelectric characteristics of PS (Jouvet 1972). On the contrary th conventional RRs have been characterized as signs of the electrocortical synchronizatio It seems that also the modified type of RRs encountered during paradoxical sleep an described in the earlier (Lehtinen and Valleala 1969) and present studies is of the sam quality It is obvious, though not yet experimentally verified, that PGO wave activity picke up from the pontine reticular formation has the same type of relationship with RRs. I fact, that region is one of likely sites of interaction of these two factors. That type of interac tion could be possible provided that the effects of the repetitive thalamic stimulation ca reach the critical brain stem region, which could happen in two principal ways, directl (Schlag and Faidherbe 1961) or indirectly *e.g* via the cortical motor area and the respecti descending tracts (Lehtinen 1972) The possibility that the lateral geniculate nuclei hav been influenced by intrathalamic connexions cannot be excluded. However it seems un likely because of the fact that the scarcity of PGO waves during RRs is clearly noticeabl not only in the ipsilateral but also in the contralateral hemisphere with respect to the sit of the thalamic stimulation. The most likely explanation is that the desynchronizing tendenc of the PGO wave mechanism is the primary factor influencing the non-specific thalamo cortical projection system, and thus preventing the appearance of RRs.

Repetitive stimulation of the non-specific thalamus can have behavioral effects like a cessation of the on-going motor activity in an alert animal (Hunter and Jasper 1949 Rougeul et al 1967). Correspondingly during PS, there are no rapid eye movements in the presence of RRs elicited by such a thalamic stimulation. In the present study it was shown that also central cerebral mechanism—in this case PGO wave activity which is closely connected with the phasic motor phenomena typical to PS—can interact with RRs.

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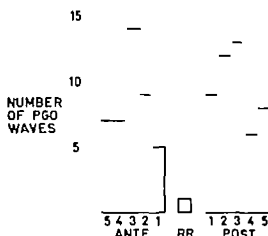


Fig. 3 Number of PGO waves in both the late geniculate nuclei during a PS episode of 3 m length. The repetitive stimulation of the left cen median nucleus was going on the whole PS episod eliciting totally ten RRs, the average length of wh amounted to 1.3 s. PGO waves were sampled duri these RRs and in five 1 s periods before (ANTE) an after (POST) RRs. For details, see text.

Fig. 3 shows the number of PGO waves in both the lateral geniculate nuclei during a PS episode of 3 min length The repetitive stimulation of the center median nucleus was going on the whole PS episode eliciting totally ten RRs, the average length of which amounted to 1.3 s. The time immediately preceding and following every RR of the sample was divided into five 1 s periods and the total number of PGO waves occurring in both lateral geniculate nuclei was determined separately for each period and during RRs. Fig. 3 shows that only once did a PGO wave occur during RR The typical series of PGO waves at a rate of 4–7 Hz never occurred during RRs.

Discussion

The results of the present study point out that PGO waves and RRs are aspects of opposite physiological tendencies. Generally PGO waves refer to an increase and RRs to a decrease in the level of activation. The electrocortical desynchronization and the PGO wave activity are among the main bioelectric characteristics of PS (Jouvet 1972). On the contrary the conventional RRs have been characterized as signs of the electrocortical synchronization. It seems that also the modified type of RRs encountered during paradoxical sleep and described in the earlier (Lehtinen and Valleala 1969) and present studies is of the same quality It is obvious, though not yet experimentally verified, that PGO wave activity picked up from the pontine reticular formation has the same type of relationship with RRs. In fact, that region is one of likely sites of interaction of these two factors. That type of interaction could be possible provided that the effects of the repetitive thalamic stimulation can reach the critical brain stem region which could happen in two principal ways directly (Schlag and Faidherbe 1961) or indirectly *e.g.* via the cortical motor area and the respective descending tracts (Lehtinen 1972). The possibility that the lateral geniculate nuclei have been influenced by intrathalamic connexions cannot be excluded However it seems unlikely because of the fact that the scarcity of PGO waves during RRs is clearly noticeable not only in the ipsilateral but also in the contralateral hemisphere with respect to the site of the thalamic stimulation The most likely explanation is that the desynchronizing tendency of the PGO wave mechanism is the primary factor influencing the non-specific thalamocortical projection system, and thus preventing the appearance of RRs.

1977 b). This was produced by progressive coronary artery stenosis and was accompanied by a 20% decrease in diastolic coronary flow, an unchanged systolic flow and a relative endocardial hypoperfusion. The distribution of coronary blood flow was estimated from the activity concentrations of Xe-133 in tissue blocks after bolus injection of the isotope into the aortic root. The reduced diastolic coronary blood flow produced by the stenosis might facilitate diffusion of xenon from vessels supplying the endocardial region during their passage through the epicardial part of the wall. This would result in activity concentrations which would not be proportionate to flows. Downey *et al.* (1975) have suggested that when perfusion pressure is reduced below a critical level stress relaxation of the subendocardial vasculature occur too slowly for adequate subendocardial perfusion. This would result in a lower endo- than epicardial blood flow reserve but only at decreased perfusion pressures.

In order to avoid substantial reductions of total coronary blood flow and perfusion pressure coronary vasodilatation was produced in a group of dogs by progressive hemodilution, until the diastolic reactive hyperemic response to 10 s occlusion of the left circumflex coronary artery just disappeared. In accordance with reports concerning experimental coronary artery stenosis (Reneman and Spencer 1972, Van Der Meer 1972, Bagger 1977 b) coronary vasodilatation was considered at a maximum when the diastolic reactive hyperemic response was abolished. At this degree of hemodilution ("optimum" hemodilution) the distribution of coronary blood flow in the left ventricular wall was estimated by measuring tissue activity concentrations of Xe 133 and radioactive microspheres injected as a bolus into the aortic root.

Occlusion of a coronary artery for 10 s results in a maximum vasodilatation peripheral to the occlusion (Olsson and Gregg 1965, Khouri, Gregg and Lowensohn 1968, Van Der Meer 1972). Furthermore, the occlusion leads to depression of myocardial contractility and systolic intramyocardial pressure (Van Der Meer 1972). In order to evaluate the influence of these changes on the distribution of coronary blood flow the left coronary artery was occluded for 10 s in another group of dogs. In this group myocardial activity concentrations were measured after injection of Xe 133 and radioactive microspheres at peak diastolic reactive hyperemia.

Methods

20 mongrel dogs of both sexes (24–49 kg) were anesthetized with pentobarbital sodium 25 mg kg^{-1} i.v. Ventilation was maintained with atmospheric air (0.275 l kg^{-1} min^{-1}) through an endotracheal tube by means of a Servoventilator-900 (Siemens-Elema).

Local anesthetic (Lidocaine-norepinephrine 1%, 5 ml) was injected into both inguinal regions and the left and right femoral arteries and right femoral vein were exposed. Ducor® "pig-tail" and left coronary artery catheters were introduced through the left and right femoral arteries respectively. Under fluoroscopy the tip of the "pig-tail" catheter was positioned in the left ventricle for measurements of diastolic pressure. The tip of the left coronary artery catheter was positioned in the aortic root without pointing at the region of the left coronary artery. This catheter was used for measurements of aortic pressure and injection of tracers. ECG was recorded in standard limb lead I or II. After injection of local anesthetic into the thoracic wall a positive end expiratory pressure of 5 cm of water was established to prevent collapse of the lungs. Thoracotomy was performed on the left side and the pericardium was opened. The bifurcation of the left coronary artery was exposed by blunt dissection. A close-fitting electromagnetic flow transducer was placed on the circumflex branch and connected to a square-wave flowmeter (Nycotron type 373-S or 376). ECG, aortic pressure and left ventricular diastolic pressure were recorded continuously on an oscilloscope and for two periods, immediately after positioning the catheters and after exposing the left coronary ar-

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Distribution of maximum coronary blood flow in the left ventricular wall of anesthetized dogs

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Abstract

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Blood flow i the circumflex branch of the left coronary artery was recorded by electromagnetic flowmeter In group of dogs p ogressive hemodilution was performed until the diastolic reactive hyperemic response to 10 s occlusion of the circumflex branch disappeared ("optimum hemodilution). At this degree of hemodilution the distribution of blood flow in the left ventricular free wall was evaluated by measuring tissue activity concentrations of Xe-133 and radioacti e microspheres after bolus injection into the aortic root. Optimum hemodilution was accompanied by a sixfold increase in systolic coronary flow a 3-fold increase in diastolic flow and a relative ndocardial hypoperfusion. These results indicate that the endocardial blood flow reserve is lower than the epicardial. This conclusion is supported by the influence of spontaneous oscillations of arterial blood pressure (Traube-Hering waves) on systolic and diastolic coronary blood flows before and during "optimum hemod lution. In another group of dogs maximum coronary vasodilatation was produced by occlusion of the left coronary artery for 10 s. In this group the distribution of Xe-133 and radioactive microspheres were measured afte bolus njection i to the aortic root at peak diastolic reactive hyperemia. The epi- and endocardial distribution of both Xe-133 and microspheres was uniform in the left ventricular wall indicating uniform flow t these regions. This might be explained by an increased endocardial perfusion during systole due to loss of myocardial contractility r by a decline towards resting level in epicardial flow t the time of injection, corresponding t a shorter duration of the hyperemic period in the epi- than endocardial region

Myocardial uptake of different diffusible tracers have indicated that blood flow per unit weight is uniform in the left ventricular wall of normal anesthetized dogs (Moir and DeBra 1967 Griggs and Nakamura 1968 Bagger 1972) This has been confirmed in some expts with radioactive microspheres of less than 15 µm in diameter (e.g Becker Fortuin and Pitt 1971) Downey and Kirk (1974) have shown that systolic coronary flow mainly supplies the epicardial part of the left ventricular wall. Therefore, uniform flow indicates that diastolic flow is larger and diastolic vascular resistance smaller in the endo- than epicardial region. As a consequence the blood flow reserve might be lower endo- than epicardially

The relative epi- and endocardial blood flow reserves have previously been evaluated by the distribution of coronary blood flow at maximum coronary vasodilatation (Bagger

TABLE I. Hemodynamic and respiratory parameters (mean ± SD) in the groups of dogs submitted to hemodilution or reactive hyperemia. c: control. d: after isolation of the coronary arteries. h: at "optimum" hemodilution or peak reactive hyperemia. LVDP: left ventricular diastolic pressure. Hgb: hemoglobin concentration. Ht: hematocrit.

	Aortic pressure mmHg		Heart rate min^{-1}	LVDP mmHg	Hgb g 100 ml^{-1}	Ht	PO_2 mmHg	PCO_2 mmHg	pH
	Systolic	Diastolic							
"Optimum" hemodilution and occlusion of the left anterior descending branch (group D_1) (N = 6)									
c	129 ± 35	98 ± 20	83 ± 20	6 ± 4	13.3 ± 1.3	44 ± 5	95 ± 9	26 ± 3	7.40 ± 0.04
d	134 ± 39	112 ± 34	130 ± 29	3 ± 4			97 ± 7	27 ± 4	7.38 ± 0.03
h	128 ± 33	91 ± 26	146 ± 26	6 ± 6	3.3 ± 0.6	11 ± 2	106 ± 9	29 ± 4	7.26 ± 0.12
"Optimum" hemodilution only (group D_2) (N = 4)									
c	129 ± 18	104 ± 20	84 ± 33	9 ± 3	12.2 ± 1.9	39 ± 5	92 ± 9	28 ± 2	7.43 ± 0.05
d	144 ± 18	121 ± 16	158 ± 28	7 ± 5					
h	136 ± 23	104 ± 26	160 ± 21	9 ± 2	4.1 ± 0.4	14 ± 2	97 ± 15	31 ± 2	7.34 ± 0.05
Peak reactive hyperemia (group R) (N = 9 unless otherwise stated)									
c	127 ± 14	98 ± 15	92 ± 23	8 ± 2	13.8 ± 1.1 N = 4	45 ± 4 N = 4	90 ± 9	31 ± 6	7.40 ± 0.05
d	140 ± 20	116 ± 17	160 ± 30	5 ± 4			97 ± 4 N = 4	32 ± 4 N = 4	7.35 ± 0.02 N = 4
h	129 ± 25	101 ± 24	171 ± 35	6 ± 4 N = 8			94 ± 8 N = 8	31 ± 7 N = 8	7.38 ± 0.02 N = 8

mean values of measurements performed immediately after introduction of the catheters (c), after isolation of the left coronary artery (d) and at "optimum" hemodilution or peak reactive hyperemia (h). "Optimum" hemodilution was accompanied by decreases in hemoglobin concentration and hematocrit to 1/3–1/4 of the initial values. In group D_2 no changes were observed in the hemodynamic parameters after occlusion of the left anterior descending branch.

Fig. 1 shows the ECG, left ventricular diastolic and aortic pressures and the instantaneous flow in the left circumflex branch before, during and after 10 s occlusion of the branch. The recordings are before the start of hemodilution (A), at a dilution which reduced the diastolic RH-response (B), and at "optimum" hemodilution (C). The figure illustrates that "optimum" hemodilution was accompanied by a relatively larger increase in minimum flow during systole ("systolic" flow) than in maximum flow during diastole ("diastolic" flow). For the "optimum" hemodiluted dogs of group D "systolic" flow increased by a factor 5.7 (range 3.7–8.0), while "diastolic" flow increased by a factor 3.3 (range 2.1–4.6). The figures are significantly different (p < 0.0001 by method of paired comparison).

Fig. 2 shows the distribution of microspheres in one dog before hemodilution (control) and of microspheres and Xe 133 in the same dog at "optimum" hemodilution and occlusion of the left anterior descending branch. The slices are numbered in the direction from the apical region towards the base of the heart. The activity concentrations of the tissue blocks of each slice are plotted in the direction from the area supplied by the left anterior descending branch towards that supplied by the circumflex branch. Before hemodilution the distribution of microspheres is uniform. At "optimum" hemodilution the levels of activities are very low

tery also on a 4-channel ink jet recorder (Mingograf 34 Elema Schonander). After application of the flow transducer ECG pressures and the instantaneous flow in the left circumflex branch were recorded continously on this recorder

11 dogs (group D) were submitted to hemodilution. In this group a snare for 10 s occlusions was placed distal to the flow transducer The left carotid artery was exposed and a Bardec catheter was introduced The tip of a polyethylene catheter was positioned in the inferior caval vein via the right femoral vein. Progressive hemodilution was performed replacing blood by 10 % dextran (mol. wt. 40.000) in 0.9 % saline (Rheomacrodex®) during frequent recordings of the reactive hyperemic response (RH-response) to 10 s occlusion of the circumflex branch. Initially 10–25 ml of blood per kg body weight were drawn from the carotid artery and replaced by the same amount of dextran solution infused into the inferior caval vein. With increasing coronary blood flow and decreasing RH response the infused volume of dextran was reduced to 40–100 ml between each recording of the RH response. In 10 dogs hemodilution was continued until the diastolic RH-response just disappeared ("optimum hemodilution). This was obtained in 84–278 min. In 6 of these dogs (group D) the anterior descending branch of the left coronary artery was occluded at its origin, Xe-133 (0.65 mCi in 2.4 ml saline) (one dog) or a mixture (vol. 3.0–5.5 ml) of Xe-133 (1.4–2.0 mCi) and radioactive microspheres (13–22 μCi Ce-141) (5 dogs) was immediately injected as a bolus into the aortic root and the heart excised at once. The time interval between start of injection and disappearance of the ECG was 4–7 s. Control injection of microspheres (39–69 μCi Cr 51 in 5 ml 10 % dextran) into the aortic root was performed prior to hemodilution I the same 5 dogs. In other 4 dogs (group D_0) submitted to optimum hemodilution occlusion of the left anterior descending branch was omitted. Xe-133 dissolved in saline (0 65–1.96 mCi in 2.4–2.9 ml) was injected as a bolus into the aortic root and the heart excised at once In this group 4–6 s elapsed between start of injection and disappearance of the ECG. In the last dog of group D hemodilution was continued beyond the disappearance of the diastolic RH-response until an increasing left ventricular diastolic pressure was observed. Then Xe-133 (0.84 mCi in 2.4 ml) was injected as a bolus into the aortic root and the heart excised at once The time interval between start of injection and disappearance of the ECG was 6 s.

9 dogs (group R) without hemodilution were used for measurements of flow distribution during peak diastolic reactive hyperemia. A loose l gature was placed around the main stem of the left coronary artery for occlusion. Xe-133 (0.65–1 62 mCi in 1 4–3 4 ml) (5 dogs) or a mixture (vol. 2.9–5.0 ml) of Xe-133 (0.78–1 66 mC) and radioactive microspheres (11 13 μCi Ce-141) (4 dogs) was injected as a bolus into the aortic root at peak diastolic coronary flow after 10 s occlusion of the left coronary artery and the heart excised at once. The time interval between start of injection and disappearance of the ECG was 3–14 s.

After introduction of the catheters, before hemodilution and immediately before injection of tracers oxygen and carbon dioxide tensions and the pH of arterial blood were measured. In all dogs submitted to hemodilution and in 4 dogs of group R additional measurements of hematocrit and hemoglobin concentration were performed

Polystyrene microspheres (3M Company) labelled with Cr 51 or Ce-141 w th diameter of 9 ± 1 (SD) μm and a density of 1.23 g cm^{-3} were used for the expts. They were obtained as 1 mCi of nuclide in 10 ml 10 % dextran containing 0.05 % polyoxyethylene 80-sorbitan mono-oleate (Tween-80) to minimize clumping. The specific activities were about 35 mCi g^{-} and 10 mCi g^{-} for spheres labelled with Cr 51 and Ce-141 respectively The number of microspheres per injection was 6 10^{6} 9 10^{6} No changes in the hemodynamic parameters were observed after the injections.

After excision of the heart the previously described procedure was followed (Bagger 1972). In brief the heart was frozen in mixture of dry ice and iso-pe tane (−75°C). The ape was removed and the heart was sliced at right angles to th long xis. From each slice the left ventricular free wall was divided into blocks. Each block was subdivided further into epi- and endocardial halves and placed in previously weighed, cooled, closed t bes. The activities were counted in well-type scintillation detector with the windows of its two channels adjusted around the 320 keV peak of Cr 51 and the 81 k V peak of Xe-133. The tubes were weighed again and the caps were removed for 24 h to allow xenon to escape The activities were then counted with the windows adjusted round the 320 k V peak of Cr 51 and the 145 keV peak of Ce-141 The activities of each isotope were calculated and expressed as cps 100 mg^{-} or cpm 100 mg^{-}

Results

Table I shows the hemodynamic and respiratory parameters measured in the ten dogs of group D subjected to "optimum" hemodilution and in all dogs of group R The figures are

CONTROL

OPTIMUM HEMODILUTION

Fig. 2. The distribution of microspheres in epi- and endocardial halves of 3 slices of the left ventricular wall of one dog before hemodilution (control) and of microspheres and Xe-133 in the same slices at "optimum" hemodilution and occlusion of the left anterior descending branch. The activity concentrations of the tissue blocks of each slice are plotted in the direction from the anterior descending (l.a.d.) towards the circumflex supplied area.

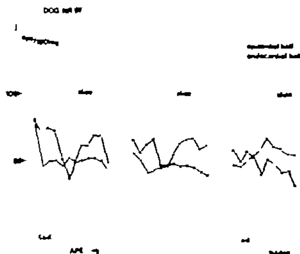


Fig. 3. The distribution of Xe-133 in the left ventricular wall of one dog plotted as in Fig. 2. Xenon was injected at "optimum" hemodilution and unimpeded flow in the left anterior descending branch.

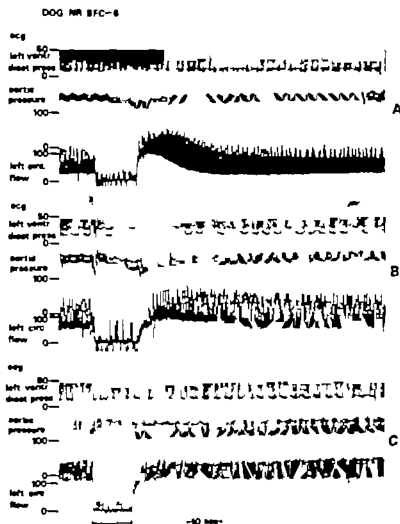


Fig. 1 ECG left ventricular diastolic and aortic pressures (mmHg) and the instantaneous flow in the left circumflex branch (arbitrary units) before during and after occlusion for 10 s. A before hemodilution, B at a dilution which reduced diastolic reactive hyperemic response C at optimum hemodilution.

in the area supplied by the occluded left anterior descending branch. In the region supplied by the circumflex branch the levels of activities are significantly higher and furthermore, the activities of both microspheres and Xe 133 are substantially higher in the epi- than endocardial region. Table II shows the mean ratio of epi- to endocardial activity concentrations of microspheres before hemodilution and of microspheres and Xe 133 at "optimum" hemodilution and occlusion of the left anterior descending branch. The ratio of microsphere activities in the circumflex area is significantly higher at "optimum" hemodilution than the corresponding control value ($p < 0.01$ by method of paired comparison). The ratios of microsphere activities and Xe 133 are not significantly different at "optimum" hemodilution ($p > 0.05$ by method of paired comparison).

Fig. 3 shows the distribution of Xe-133 in three slices of the left ventricular wall of an "optimum" hemodiluted dog with unimpeded flow in the left anterior descending branch. The activity concentrations are plotted as in Fig. 2 and demonstrate a higher epi- than

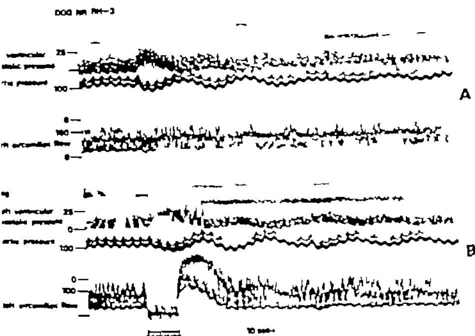


Fig. 3 Traube–Hering waves generated spontaneously (A) and during 10 occlusion of the left coronary artery (B).

paired comparison). Compared to the ratio obtained in hemodiluted dogs with occluded left anterior descending branch (1.81 ±0.11) the corresponding ratio is significantly lower (p 0.05).

The last dog of group D was hemodiluted beyond the disappearance of the diastolic RH-response and until signs of left ventricular insufficiency appeared. Left ventricular diastolic pressure increased from 3 (before the onset of hemodilution) to 47 mmHg, aortic systolic and diastolic pressures decreased from 161 to 106 mmHg and from 136 to 86 mmHg respectively. "Diastolic" coronary flow was reduced to control level, while "systolic" flow as twice as high as the initial value. Fig. 4 shows the distribution of activity in the left ventricular free wall of this dog plotted as in Fig. 2. The ratio of epi- to endocardial activities a 3.11 ±0.15 (SE), which is significantly higher than in the circumflex supplied area of "optimum" hemodiluted dogs with occlusion of the left anterior descending branch ($p < 0.01$).

In group R "systolic" and "diastolic" flows were increased by factors 4.6 (range: 3.2–7.0) and 2.6 (range: 1.8–4.0) respectively at peak reactive hyperemia. These factors are significantly different (p 0.005 by method of paired comparison). Table II shows the mean ratios of epi- to endocardial activity concentrations in this group. The ratio for Xe 133 is significantly higher in the circumflex than in the left anterior descending area ($p < 0.05$ by method of paired comparison). Compared to the ratio in the circumflex area of hemodiluted dogs with unimpeded flow in the left anterior descending branch (1.38 ±0.06) the corresponding ratio in group R is significantly lower ($p < 0.02$). The ratio of microsphere ac

TABLE II Ratios of epi- to endocardial activity concentrations of Xe-133 and radioactive microspheres in the left ventricular free wall (mean ± SE). l.a.d. and circumflex areas regions supplied by the left anterior descending and circumflex branches respectively

	l.a.d. area	Circumflex area	Whole left ventricular wall
Control (group D_1).			
microspheres (N = 5)	0.86 ± 0.05	0.92 ± 0.05	0.88 ± 0.05
"Optimum" hemodilution and occlusion of the left anterior descending branch (group D).			
microspheres (N = 5)		1.72 ± 0.14	
Xe-133 (N = 6)		1.81 ± 0.11	
"Optimum" hemodilution only (group D)			
Xe-133 (N = 4)	0.98 ± 0.07	1.38 ± 0.06	1.18 ± 0.07
Peak reactive hyperemia (group X).			
microspheres (N = 4)	0.85 ± 0.16	0.90 ± 0.10	0.88 ± 0.11
Xe-133 (N = 9)	0.90 ± 0.06	1.05 ± 0.07	0.97 ± 0.05

endocardial level of activity in the area supplied by the circumflex branch. In all four dogs subjected only to "optimum" hemodilution the activity concentration was significantly higher in the epi- than endocardial region but only in areas supplied by the circumflex branch. Table II shows the mean ratios of epi- to endocardial activity concentrations of Xe 133 in these four dogs. The ratio is significantly higher in the region of circumflex supply than in the region supplied by the left anterior descending branch ($p < 0.005$ by method of

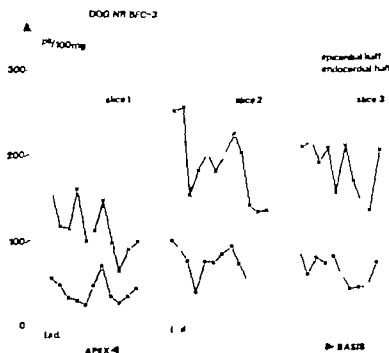


Fig. 4. The distribution of Xe-133 in the left ventricular wall of one dog plotted as in Fig. 2. Xenon was injected at a degree of hemodilution beyond "optimum" causing left ventricular insufficiency

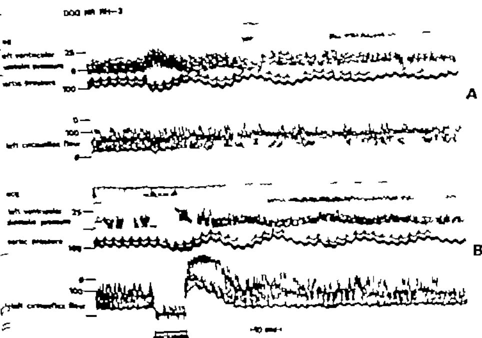


Fig. 3 Traube–Hering waves generated spontaneously (A) and during 10 occlusion of the left coronary artery (B).

paired comparison). Compared to the ratio obtained in hemodiluted dogs with occluded left anterior descending branch (1.81 ± 0.11) the corresponding ratio is significantly lower (p 0.05).

The last dog of group D was hemodiluted beyond the disappearance of the diastolic RH-response and until signs of left ventricular insufficiency appeared. Left ventricular diastolic pressure increased from 3 (before the onset of hemodilution) to 47 mmHg, aortic systolic and diastolic pressures decreased from 161 to 106 mmHg and from 136 to 86 mmHg respectively "Diastolic" coronary flow was reduced to control level, while "systolic" flow was twice as high as the initial value. Fig. 4 shows the distribution of activity in the left ventricular free wall of this dog plotted as in Fig. 2. The ratio of epi- to endocardial activities as 3.11 ± 0.15 (SE), which is significantly higher than in the circumflex supplied area of optimum hemodiluted dogs with occlusion of the left anterior descending branch ($p < 0.01$).

In group R "systolic" and "diastolic" flows were increased by factors 4.6 (range: 3.2–7.0) and ..6 (range: 1.8–4.0) respectively at peak reactive hyperemia. These factors are significantly different ($p < 0.005$ by method of paired comparison). Table II shows the mean ratios of epi- to endocardial activity concentrations in this group. The ratio for Xe 133 is significantly higher in the circumflex than in the left anterior descending area ($p < 0.05$ by method of paired comparison). Compared to the ratio in the circumflex area of hemodiluted dogs with unimpeded flow in the left anterior descending branch (1.38 ± 0.06) the corresponding ratio in group R is significantly lower ($p < 0.02$). The ratio of microsphere ac

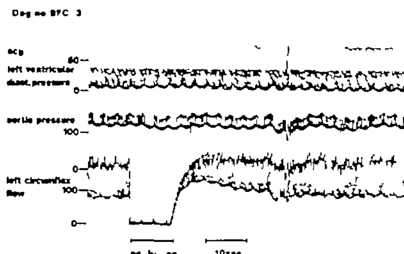


Fig. 6. Reactive hyperemic response in a hemodiluted dog with submaximum coronary vasodilatation. Note that the Traube–Hering waves only influence "diastolic" coronary blood flow

tivity concentrations in areas supplied by the left anterior descending and circumflex branches are not significantly different. Furthermore, they are not significantly different from the corresponding ratios for Xe 133

In some of the dogs of both groups (D and R) periods of fluctuations in arterial blood pressure appeared spontaneously and during the 10 s occlusions. These fluctuations, which had a frequency lower than that of the respiratory changes, are generally called vasomotor or Traube–Hering waves (TH waves) (*e.g.* Guyton and Harris 1951). The present expts.

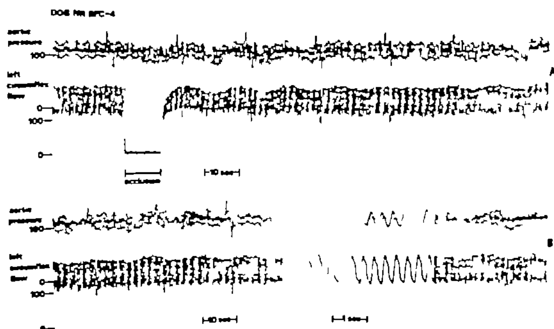


Fig. 7. Traube–Hering waves generated during 10 s occlusion of the left circumflex branch (A) and spontaneously (B) in a dog with "optimum" hemodilution. The pressure waves are reflected in both "systolic" and "diastolic" circumflex flow

eem to indicate a relation between the influence of these fluctuations on coronary blood low and the degree of coronary vasodilatation. Therefore, the expts. in which they appeared will be examined more closely in this report. Fig. 5 shows TH-waves generated spontaneously (A) and during 10 s occlusion of the left coronary artery (B) in a normal, anesthetized dog. Although changes in arterial blood pressure produced by respiration were accompanied by oscillations in "systolic" coronary flow the TH-waves in A, apart from the initial, steep pressure decrease, did not influence "systolic" or "diastolic" coronary flow. When TH waves were present during reactive hyperemia (B), they were accompanied by oscillations in "diastolic" coronary flow while influence on "systolic" flow was not observed. In Fig. 6 coronary blood flow was recorded in a dog hemodiluted to a very small diastolic RH response, indicating almost maximum dilated coronary vessels. Also in this dog the TH-waves only produced fluctuations in "diastolic" coronary flow. Finally Fig. 7 shows that TH-waves at "optimum" hemodilution were reflected in both "diastolic" and "systolic" coronary blood flows.

Discussion

The activity concentrations of microspheres injected into the aortic root before hemodilution showed a uniform distribution in the left ventricular wall. This is in accordance with other reports about radioactive microspheres with a mean diameter of less than 15 μm, injected into the left atrium (e.g. Buckberg et al. 1975) and with the distribution of Xe-133 after a bolus injection into the aortic root of normal, anesthetized dogs (Bagger 1977 a).

At "optimum" hemodilution and occlusion of the left anterior descending branch the activity concentrations of both Xe-133 and microspheres were significantly higher in the epi- than endocardial region. Furthermore, there was no difference between the ratios of epi- to endocardial activity concentrations of the two tracers. This seems to indicate that wash out of Xe-133 in the short period between injection of the isotope and cessation of coronary flow was negligeable. Occlusion of the left anterior descending branch without hemodilution does not influence the distribution of Xe 133 in the circumflex supplied area (Bagger 1977 b). Therefore, the results indicate that the blood flow reserve is smaller in the endo- than epicardial region. This might explain why hemodilution was accompanied by a smaller increase in "diastolic" than "systolic" coronary blood flow. The present expts. were performed at maximum coronary vasodilatation accompanied by increased coronary blood flow and presumably modest decrease in perfusion pressure of the circumflex supplied region. This makes it unlikely that increased fractional loss of isotope from vessels supplying the endocardium during their passage through the epicardial area or a too slow stress relaxation of the subendocardial vasculature were the only reasons for the lower endo- than epicardial activity previously found during maximum vasodilatation with reduced coronary flow and perfusion pressure (Bagger 1977 b).

Because of the short or even absent main stem of the left coronary artery in dogs the flow transducer was placed on the left circumflex branch. Thus hemodilution was continued until the vessels from this branch were dilated at a maximum defined by disappearance of the diastolic reactive hyperemic response. Spencer and Denison (1959) stated that the reliability of pulsatile flow measurements obtained with electromagnetic flowmeter depends on a good

contact between the electrodes and the vessel This can only be achieved if the constricts the vessel, reducing its diameter about 15% (Meisner Messmer and Hagl 19 Such a stenosis will probably not cause a significant reduction in blood flow under nor conditions due to autoregulatory peripheral vasodilatation, but it will reduce the blood f reserve of the supplied area. This will result in maximum vasodilatation of the area lower degree of hemodilution than otherwise necessary In the present expts. hemodilu was continued just until maximum vasodilatation was obtained in the circumflex supp area Assuming a small stenosis of the circumflex branch owing to the flow transducer dilatation of the vessels from the left anterior descending branch was probably not a maximum, when the diastolic RH-response in the circumflex supplied area was abolish This might explain the normal distribution of Xe-133 in the region supplied by the anterior descending branch of hemodiluted dogs with unrestricted flow in this artery

Progressive stenosis of the circumflex branch until maximum vasodilatation periphe to the stenosis results in the dog in a collateral flow which is able to prevent endocar hypoperfusion (Bagger 1977 b) In the circumflex supplied region of "optimum" hemodilu dogs with unrestricted flow in the left anterior descending branch the ratio of epi- to en cardial activity concentrations was lower than that of the group with occlusion of the a branch. This might indicate that collateral flow also participates in perfusion of the circu flex supplied area at "optimum" hemodilution However it was not able to prevent a relat endocardial hypoperfusion.

Provenza and Scherlis (1959) examined dog hearts and found that vessels supplying endocardial region were of larger diameter than those supplying the epicardium. Accordin Downey *et al* (1975) found a 40% higher endo- than epicardial activity of radioact microspheres in perfused fibrillating dog hearts with maximum dilated coronary vess while Cutarelli and Levy (1963) found a uniform distribution of Rb-86 in fibrillating hea as long as the intraventricular pressure remained at a normal diastolic level The pres results indicate that even if there is a smaller endo- than epicardial diastolic vascular resistan at maximum vasodilatation, it can not fully compensate for the lower endo- than epicard perfusion during systole.

Downey *et al* (1975) found that coronary vasodilatation generated by infusion of pa verine into the left circumflex branch resulted in identical epi and endocardial activity co centrations of radioactive microspheres. This contrast to the present expts. might be due a difference between the degree of vasodilatation produced by papaverine and hemodiluti hypoxia.

Salisbury Cross and Rieben (1963) and Hirshorn and Kaiser (1970) found that increas left ventricular end diastolic pressure resulted in endocardial hypoperfusion presumab because of a decreased diastolic driving pressure. In the present expts. left ventricular dia tolic pressure was not influenced by "optimum" hemodilution (Table I) However in o expt. in which hemodilution was continued beyond "optimum" until left ventricular diasto pressure increased, the relative endocardial hypoperfusion was accentuated and now prese in both areas supplied by the two main branches of the left coronary artery

Cyclic changes in arterial blood pressure at a frequency of 2–4 per min, so-called Traub Hering waves, did not influence the coronary blood flow when they appeared spontaneous

in normal dogs (Fig. 5 A). As in the same recordings respiratory changes in arterial blood pressure were reflected in "systolic" coronary blood flow the lacking influence of the TH waves seems to indicate synchronous fluctuations in coronary vascular resistance. These fluctuations would correspond to changes in peripheral vascular resistance caused by a varying sympathetic tone (Aalkjær 1935 Green, Lewis and Nickerson 1943). The fluctuating coronary vascular resistance is probably not due to autoregulation as this does not occur without any change in flow (Driscol, Moir and Eckstein 1964). More likely it is a result of varying sympathetic influence directly on the coronary vessels. At maximum coronary vasodilatation elicited by blood dilution the TH-waves were reflected in both "systolic" and "diastolic" coronary blood flows (Fig. 7) indicating a disappearance of fluctuations in coronary vascular tone. In Fig. 6 TH-waves in a dog with submaximum coronary vasodilatation were accompanied by a fluctuating "diastolic" coronary flow while "systolic" flow was uninfluenced. This finding may be due to maximum vascular relaxation in the region mainly perfused during diastole (the endocardial part of the wall) and incomplete relaxation (and maintained responsiveness to sympathetic stimuli) in the region also perfused during systole (the epicardial part). This is in accordance with the conception of a smaller blood flow reserve in the endo- than epicardial region.

The results obtained after injection of Xe 133 and microspheres at peak diastolic reactive hyperemia showed a uniform distribution of both tracers in the epi- and endocardial regions of the left ventricular wall. This seems to indicate a uniform flow to these regions. Coronary occlusions of 10 s duration reduce myocardial contractility and systolic intramyocardial pressure (Van Der Meer 1972). Thus the uniform flow might be due to an improved endocardial perfusion during systole. This might also explain the larger increase in "systolic" than "diastolic" flow at peak reactive hyperemia. However this larger increase might also be explained by the larger epi- than endocardial blood flow reserve. Reneman and Spencer (1972) found that after 10 s occlusion of the left anterior descending coronary artery "systolic" reactive hyperemic flow reached its peak value sooner and declined faster than "diastolic" flow. The present expts. confirm these observations (*e.g.* Fig. 1 A). This might indicate a larger peak hyperemic flow and a shorter lasting hyperemic period in the epi- than endocardial region. It is therefore possible that at the time of the bolus injection, *i.e.* at peak diastolic reactive hyperemia, the reactive hyperemia, while still maximum in the endocardial region, might have declined in the epicardial. This might explain the uniform flow at peak diastolic reactive hyperemia and also why TH-waves present during reactive hyperemia only influence "diastolic" coronary flow detectably.

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The influence of scrotal warming on testicular blood flow and endocrine function in the rat

By

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Abstract

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The effects of local heating on testicular and epididymal vascular resistance in sodium pentobarbitone anesthetized rats was measured with microsphere technique. When exposing the left scrotum to 33 and 37°C for 30 min, no significant effects on blood flows were observed in comparison to those of the right side. Exposure to 41°C caused significant ($p < 0.05$) decrease in vascular resistance of both testes and epididymides. The response was more pronounced at 43°C. The Leydig cell function, as judged from the testosterone concentrations in plasma and testicular tissue after LH stimulation, was significantly ($p < 0.01$) depressed at 41 and 43°C. It was concluded that the impaired Leydig cell function was unrelated to testicular blood flow.

In most mammals the scrotal temperature is lower than that of the peritoneal cavity and this difference in temperature is a prerequisite for a normal spermatogenesis. Impaired testicular function in cryptorchidism (for a review see, VanDemark & Free 1970) and varicocele (Greenberg 1977) is often attributed to an increased temperature. Although it has been shown that the secretion of testosterone is impaired in the congenitally (Eik Nes 1966) and experimentally (Damber *et al.* 1978) cryptorchid testis it is not clear to what extent the Leydig cells are dependent on low temperature for a normal sensitivity to gonadotrophic stimulation.

A possible explanation to the heat induced impairment of testicular function is disturbance of nutrition and oxygenation (Ewing & VanDemark 1963, Waites & Setchell 1964) and thus, variations in testicular blood flow may be of importance. Several reports in various species indicate a moderate increase in testicular blood flow at temperatures above 40°C but hardly any response at temperature below this level (Glover 1966, Waites *et al.* 1968, Waites *et al.* 1973, Setchell *et al.* 1966)). It has been shown that the experimentally cryptorchid testis in the rat has a relative increase in blood flow as compared to the scrotal one (Glover 1965, Damber *et al.* 1978). On the other hand, Ewing *et al.* (1963) reported a decreased flow of the perfused rabbit testis with increased temperature. A possible explanation to such discrepancies in results may be that testicular blood flow measurements are affected by the ex-

-idymides and some other organs as measured. Immediately after the injections of microspheres, the y temperature as measured through an abdominal incision. The vascular resistance of the various -ans was calculated as mean arterial blood pressure/organ blood flow per unit of weight.

Testing of the Leydig cell sensitivity to LH. Forty rats were anesthetized and the tail artery was cannulated polyethylene tube (PE 50, Intramedic Clay Adams, USA i.d. 0.6 mm, o.d. 1.0 mm) for sampling of od and i injections. Fifteen I.U. of heparin was given i.a. An arterial blood sample (0.6 ml) was then -en for determination of the basal testosterone level, immediately followed by an i.a. injection of 30 µg 1 (NIH-B10). In each rat the scrotum was immersed in thermostat regulated water bath. Immersion of , tail and hind legs was avoided. The rats were divided into 4 groups with 10 rats in each group, and the -ota were exposed to 33, 37 41 and 43°C, respectively for 30 min. Immediately after the scrotal warming body temperature was measured through an abdominal incision, and another blood sample was collected plasma testosterone determination, and the testes ere removed, decapsulated and weighed. For testi-ar testosterone analyses each testis as homogenized in 0.15 M NaCl.

Testosterone determination. All blood samples were centrifuged and plasma and tissue homogenates were -red at 20°C until analyses. The plasma samples were analyzed using radio-immunoassay previously -scribed (Damber & Janson 1978 b). From each testicular homogenate sample corresponding to 0.1 g of -ue was extracted 3 times ith 5 ml chloroform. The combined chloroform extracts were washed ith 1 ml M NaOH, then twice with 2 ml destilled ater and evaporated to dryness under stream of nitrogen in -ater bath (40°C). Testosterone determination was performed using radio-immunoassay described by -amber & Janson (1978)

Results

The body temperature in anesthetized rats was 36.9 ± 0.3 C (mean ± S.E.). The difference scrotal and abdominal temperature was 3 7 ± 0.2°C which was in agreement with the -ndings of Kormano (1967) in the conscious rat.

-ffects on unilateral scrotal warming on testicular and epididymal vascular esistance

-he warming of the left scrotum did not affect the temperature of the right testis. *Table I* -ows that local scrotal warming elicited no significant effects on body temperature, blood -essure and on the distribution of blood flow to ventral prostate, kidneys and brain.

Table II shows a significant decrease ($p < 0.05$) in vascular resistance of testes and epididy-ides at temperatures of 41 and 43°C. The effect on the epididymis was more pronounced -an that on the testis.

-ABLE I The effect of unilateral scrotal warming on mean arterial blood pressure and vascular resistance in -ous organs in anesthetized rats

-eft scrotal -mperature (-C)	No.	Body tempe-rature (°C)	Mean arterial blood pressure (mm Hg)	Vascular resistance (mmHg/ml/100 g min) Ventral prostate	Kidneys	Brain
)	6	37 1 ± 0.3	111.7 ± 4 4	5.6 ± 0.4	0 20 ± 0 02	3 0 ± 0.5
7	5	37 0 _ 0 4	110 8 ± 4 4	5 6 ± 0.7	0.27 ± 0 03	3.3 ± 0 6
1	6	37.2 ± 0.2	112.5 ± 8 4	5 6 ± 1.2	0.24 ± 0 03	2.8 ± 0 5
3	6	36 6 ± 0.3	102.5 ± 6 2	5 2 ± 0 6	0.18 ± 0 03	2.4 ± 0 7

-lues are expressed as mean ± S E.
-No significant differences between the groups for any parameter were achieved by analysis of variance.

perimental design, especially since scrotal heating above 40°C in a small animal like the r has been shown to elicit profound effects on body temperature and several cardiov parameters (Waites *et al* 1973) possibly concealing local effects on the testicular vascul (Damber & Janson 1977)

The present study was undertaken to re-evaluate the effect of local heating blood flow in the rat using the microsphere technique. In addition, the gonadotropi sensitivity of the Leydig cells was tested *in vivo* at normal and supernormal temperatu

Materials and methods

Animals. Male rats of the Sprague-Dawley strain (Anticimex Ltd, Stockholm, S eden) weighing 360–43 were kept in a controlled environment (temperature 25 C, light 05.00–19.00 h). Food and water w available *ad libitum* The anaesthetic used was sodium pentobarbitone (Nembutal, Abbott, U.K.) given i, as a single dose of 40 mg/kg.

Measurement of scrotal and abdominal temperature In 5 anesthetized rats iron-constantan thermocoupl were placed in the right scrotum and in the middle part of the abdominal cavity through small skin incisio The thermocouple was connected to a PM 421 D digitalmultimeter (Philips).

Measurements of blood flow In 4 anesthetized rats a midscrotal skin incision was performed and the l scrotal sac was introduced into a closely fitting copper basket which was surrounded by a heating jack connected to a circulatron thermostat. The heating device was kept in place by a thin rubber dome, holdin the upper part of the device just above the cranial pole of the epididymis. The scrotal temperature was re corded by a thermistor connected to a Grass Model 7 Polygraph. The blood pressure and the distributio blood flow was measured with the microsphere technique as described previously (Damber & Janson 197 The spheres used were labelled w th ^{46}Sc (initial specific activity 13.54 mCi/g) and had a diameter of 14.5±1 µm (mean size ± range). The experimental design is schematically illustrated in *Fig 1* The rats were di into 4 groups (6 rats n each group) in which the left testis was exposed to 33 37 41 and 43°C, respectiv The right testis served as a control. After a period of 30 mi the distribution of blood flow to the test

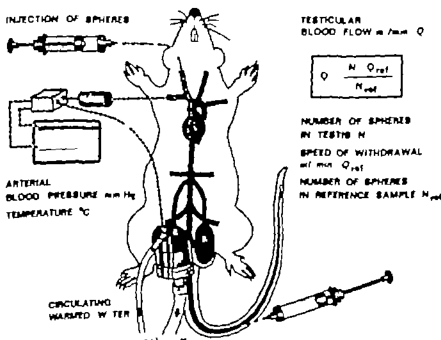


Fig. 1 Schematic illustration of the animal preparation. For details of the microsphere techn que, se Damber & Janson 1977

idymides and some other organs was measured. Immediately after the injections of microspheres, the y temperature was measured through an abdominal incision. The vascular resistance of the arious ans was calculated as mean arterial blood pressure/organ blood flow per unit of eight.

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...BLE I. The effect of unilateral scrotal warming on mean arterial blood pressure and vascular resistance in various organs in anesthetized rats.

...ft scrotal ...mperature ...C)	No.	Body tempe- rature (°C)	Mean arterial blood pressure (mmHg)	Vascular resistance (mmHg/ml/100 g min) Ventral prostate	Kidneys	Brain
	6	37 1 ± 0 3	111 7 ± 4 4	5 6 ± 0 4	0.20 ± 0 02	3 0 ± 0.5
7	5	37.0 ± 0 4	110 8 ± 4.4	5 6 ± 0 7	0.27 ± 0 03	3.3 ± 0 6
1	6	37 2 ± 0 2	112 5 ± 8.4	5 6 ± 1.2	0.24 ± 0 03	2.8 ± 0 5
3	6	36.6 ± 0.3	102.5 ± 6.2	5.2 ± 0 6	0.18 ± 0.03	2.4 ± 0.7

...lues are expressed as mean ± S.E.
...o significant differences between the groups for any parameter were achieved by analysis of variance.

TABLE II The effects of a left-sided scrotal warming on testicular and epididymal vascular resis (mmHg/ml/100 g min) in anesthetized rats.

Left scrotal temperature (°C)	No	Testes			Epididymis		
		Right	Left	% Difference	Right	Left	% Differ
33	6	6.9 ± 1.4	7.6 ± 1.9	+ 5.6 ± 7.8	14.3 ± 2.3	13.7 ± 2.0	− 3.2 ± 4
37	5	7.5 ± 1..	6.8 ± 1.0	− 8.2 ± 4.3	13.8 ± 1.9	12.5 ± 1.2	− 6.8 ± 1
41	6	8.5 ± 1.5	6.3 ± 1.3	−28.6 ± 3.1	17.5 ± 3.4	10.0 ± 1.9	−41.9 ± 5
43	6	7.3 ± 1.5	3.1 ± 0.8	−56.2 ± 6.7	15.3 ± 3.4	3.1 ± 0.7	−74.8 ± 7

Values are expressed as mean ± S.E.
Significant decrease ($p < 0.05$) of vascular resistance according to Wilcoxon's paired t test

Effects of bilateral scrotal warming on the Leydig cell sensitivity to LH

The body temperature after 30 min of scrotal warming to different temperatures was 36.4 0.1 C and was not significantly different between the groups or from the basal levels (see above). The effects of scrotal warming on plasma testosterone concentration before and after LH stimulation and the testicular testosterone concentration after LH treatment are shown in *Table III*. *Fig. 2* illustrates the acute responsiveness of the testes to LH stimulation at increasing temperatures, expressed as the difference in plasma testosterone concentration before and 30 min after LH injection. The results clearly demonstrate that the Leydig cell function, expressed as the acute response to LH stimulation, is impaired at 41 and 43°C.

Discussion

The moderate response in vascular resistance to increased scrotal temperatures found in the present study confirms the findings in the rat by Waites *et al.* (1973) and those in the ram by Setchell *et al.* (1966). The experimental design to induce an increased testicular temperature did not evoke any alterations in body temperature and in any other cardiovascular parameter measured, indicating that the effect on testicular and epididymal vasculature is a loc

TABLE III Effects of scrotal warming on plasma and testicular testosterone concentration 30 min after i.a. injection of 30 μg LH

Scrotal temperature (°C)	No.	Plasma testosterone concentration (ng/ml) Before LH treatment	Plasma testosterone concentration (ng/ml) After LH treatment	Testis testosterone concentration (ng/g) After LH treatment
33	8	5.5 ± 0.9	46.1 ± 2.9	2 340 ± 85
37	8	6.2 ± 0.6	51.0 ± 3.4	2 790 ± 118
41	10	5.0 ± 0.4	21.4 ± 2.1[a]	830 ± 119[a]
43	9	5.6 ± 0.5	9.8 ± 0.4[a,b]	214 ± 21[a,b]

Values are expressed as mean ± S.E.
[a] Significant different from the groups with a scrotal temperature of 33°C and 37°C, $p < 0.01$ (Wilcoxon two-sample t-test).
[b] Significantly different from the group with a scrotal temperature of 41°C. $P < 0.01$

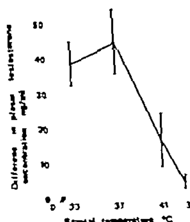


Fig. 2 Difference in plasma testosterone concentrations before and 30 min after 30 µg LH i at different scrotal temperatures. Bars indicate Mean ± Standard Deviation. significantly different from values at 33°C (p 0.01 Wilcoxon two-sample t-test). † significantly different from values at 41°C (p 0.01)

one. The finding of no demonstrable change in testicular blood flow at 37°C supports the view that the increase in relative blood flow to the cryptorchid testis is due to morphological changes rather than to vasodilatation (Damber *et al.* 1978 c). The report by Ewing & VanDemark (1964) of decreased blood flow with increased temperature in the perfused rabbit testis was not supported by the results of the present measurements *in vivo*.

It has been shown in experiments with two different species (dog: Eik Nes 1964, rat: Free & Tillson 1973, Damber & Janson 1978 a) that testosterone secretion is positively related to testicular blood flow at normal temperatures. In the present study a decrease in testicular sensitivity to LH and concomitant increase in testicular blood flow at supernormal temperatures indicate that the effect of heat on the Leydig cell function is not mediated by a primary effect on testicular perfusion as suggested by Ewing & VanDemark (1964). Collins & Lacy (1967) reported no change in interstitial steroid content after exposing rat testes to 43°C for 30 min. However it may well be that basal testosterone production is unaffected at short term exposures to heat while the capacity of the stimulated Leydig cell to produce/secrete androgens is affected as shown in the present study. It is interesting to note that the same temperature (41°C) which impairs the Leydig cell function at short term exposures is known to reduce sperm output from the rat testis (Setchell & Waites 1972) and testicular weight (Main & Waites 1977). It is tempting to speculate that the reduction in sperm output and testicular weight is due to an impaired Leydig cell function. Since it has been shown that the Leydig cell function is impaired in the experimentally cryptorchid testis of the rat (Damber *et al.* 1978), it is possible that long term exposure to an abdominal temperature elicits the same type of endocrine changes as short term exposures to supernormal temperatures. The effect on testicular function following experimentally induced cryptorchidism or heat treatment appears to be dependent on the duration of exposure as well as on the temperature of exposure (VanDemark & Free 1970).

The mechanism responsible for the decreased sensitivity of the Leydig cells to LH stimulation may be heat induced changes in their metabolism. Hall (1965) found a maximal effect of LH stimulation on rabbit testicular protein and testosterone synthesis *in vitro* at 38°C, which

TABLE II The effects of a left-sided scrotal warming on testicular and epididymal vascular resistance (mmHg/ml/100 g min) in anesthetized rats.

Left scrotal temperature (°C)	No.	Testes			Epididymis		
		Right	Left	% Difference	Right	Left	% Difference
33	6	6.9 ± 1 4	7 6 ± 1 9	+ 5 6 ± 7.8	14.3 ± 2.3	13 7 ± 2.0	− 3.2 ± 4.4
37	5	7.5 ± 1..	6.8 ± 1.0	− 8.2 ± 4.3	13.8 ± 1.9	12.5 ± 1..	− 6.8 ± 8.5
41	6	8.5 ± 1.5	6.3 ± 1.3	− 28 6 ± 3.1	17.5 ± 3 4	10.0 ± 1.9	− 41.9 ± 5.3
43	6	7.3 ± 1.5	3 1 ± 0.8	− 56.2 ± 6.7	15.3 ± 3 4	3 1 ± 0.7	− 74 8 ± 7.5

Values are expressed as mea ± S.E.
Significant decrease ($p < 0.05$) of vascular resistance according to Wilcoxon s paired t-test.

Effects of bilateral scrotal warming on the Leydig cell sensitivity to LH

The body temperature after 30 min of scrotal warming to different temperatures was 36.4 0 1°C and was not significantly different between the groups or from the basal levels (se above) The effects of scrotal warming on plasma testosterone concentration before an after LH stimulation and the testicular testosterone concentration after LH-treatment a shown in *Table III* *Fig 2* illustrates the acute responsiveness of the testes to LH stimulatio at increasing temperatures, expressed as the difference in plasma testosterone concentratio before and 30 min after LH injection The results clearly demonstrate that the Leydig c function, expressed as the acute response to LH stimulation, is impaired at 41 and 43°C.

Discussion

The moderate response in vascular resistance to increased scrotal temperatures found present study confirms the findings in the rat by Waites *et al.* (1973) and those in the ram b Setchell *et al.* (1966) The experimental design to induce an increased testicular temperatu did not evoke any alterations in body temperature and in any other cardiovascular para meter measured, indicating that the effect on testicular and epididymal vasculature is a loc

TABLE III Effects of scrotal warming o plasma and testicular testosterone concentration 30 min after i.a. injection of 30 μg LH

Scrotal temperature (°C)	No.	Plasma testosterone concentration (ng/ml)		Testis testosterone concentration (g/g)
		Before LH treatment	After LH-treatment	After LH-treatment
33	8	5.5 ± 0.9	46.1 ± 2.9	2 340 ± 85
37	8	6.2 ± 0.6	51.0 ± 3.4	2 790 ± 118
41	10	5.0 ± 0.4	21 4 ± 2.1[a]	830 ± 119[a]
43	9	5.6 ± 0.5	9.8 ± 0.4[a,b]	214 ± 21[a,b]

Values are expressed as mean ± S.E.
Significant different from the groups with a scrotal temperature of 33°C and 37°C, $p < 0.01$ (Wilcoxon two-sample t-test).
[b] Significantly different from the group with a scrotal temperature of 41°C, $P < 0.01$

Measured with Krypton[85]: Effects of heat, catecholamines and acetylcholine. *Circulat. Res.* 1966. *18* 755–765.

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is the normal scrotal temperature in this species. In addition, LeVier & Spaziani (196 found in experiments with unstimulated rat testicular tissue *in vitro* an inhibition of prote synthesis concomitant with a reduction in steroid conversion, at temperatures above an below normal scrotal levels. Heat induced changes in testicular glucose metabolism may als be of importance (Setchell & Hinks 1967 Davis & Langford 1970 Deb *et al* 1977).

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Studies on calcium uptake by myometrial microsomes with particular reference to the dependence on inorganic phosphate and oxalate

By

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Abstract

BATRA S *Studies on calcium uptake by myometrial microsomes with particular reference the dependence on inorganic phosphate and oxalate* Acta physiol. scand 1978 *104* 68–7

Ca uptak by microsomes isolated f om non pregnant rabbit myometrium was pot ntiated by both i organic phosphate (P_i) and oxalate nions. Both P_i and oxalate had little ffect on the initial rate of upt but pronounced effect on the capacity of Ca upt k measured after 20 min which was greater in t presence of oxalate than that f P_i (5 mM each). The presence r bsence of sucrose in th uptake medi had a significant effect on oxalate-induced pot ntiation of Ca uptake but not on that potentiated by o that measured in the absence of either potentiating anion A part of Ca accumulated additionally und the influence of sucrose could be removed by washing microsomes with KCl

Another significant difference between the pontentiating effect of xalate and P_i was observed wh the pH of the incubation medium was varied. In the presence of oxalat the pH optimum was betwe 6 4–6.8 whereas that in it bsence or n the presence of P the optimal pH was around 7.2 Reductio in pH from 7.2 to 6.8 along with the substituti n of KCl by sucrose resulted in 3-fold increase in C uptake whe oxalate was used as the potentiating anion. The results suggest that Ca is taken up by different mechanism in the presence of oxalat th n that in its absence or when oxalate anion is substitut with inorganic phosphate.

Since the demonstration of an ATP-dependent Ca uptake in isolated microsomal an mitochondrial fractions from the myometrium (Batra and Daniel 1969 Batra and Dani 1971 a, Carsten 1969 Batra and Daniel 1971 b) Ca uptake by subcellular fractions isolate from several types of smooth muscles has been reported (see Janis and Daniel 1977 fo references) Ca uptake by the microsomal fraction of smooth muscle differed not onl quantitatively from that of skeletal muscle (Batra 1975) but certain qualitative difference in particular the lack of stimulation by oxalate of Ca uptake by smooth muscle microsome were also noted (Batra and Daniel 1971 a, Baudovin-Legros and Meyer 1973 Zelck *et a*

975 Worcel *et al.* 1976). In some other studies, however oxalate stimulation in microsomes isolated from certain smooth muscles (Fitzpatrick *et al.* 1977 Hurwitz *et al.* 1973, Ford and Hess 1975 Godfraind *et al* 1976, Raeymaekers *et al.* 1977) could be demonstrated. Although Ca uptake in some of these recent investigations (Ford and Hess 1975 Godfraind *et al.* 1976, Raeymaekers *et al.* 1977) was measured by Millipore-filtration technique (Martonosi and Feretos 1964), the retentate (material on the filter) rather than the filtrate was counted for Ca^{45}-radioactivity determinations. This modification, which has also been recently made in the measurements of mitochondrial Ca uptake (Jacobus *et al.* 1975) makes an important difference in that Ca uptake without unspecific (or loose) binding is measured. This is achieved by washing the material on the filters before the determination of the retained isotopic Ca. Furthermore, when low amounts of Ca are taken up, the reliability of the method measuring uptake directly in the retentate, rather than by the difference in the filtered and unfiltered samples, is better

Since inorganic phosphate (P_i) is a physiological anion, occurring in relatively high concentrations in the myoplasm, we have, in the present study examined its effects on Ca uptake kinetics in rabbit myometrial microsomes and compared them with those of oxalate. Not only e are able to clearly demonstrate the potentiation by P as well as by oxalate but provide some evidence showing that the two anions increase Ca accumulation by different mechanisms.

Methods

Preparation of microsomes

Non-pregnant New Zealand hite rabbits weighing between 2.7 to 3.2 kg ere killed by cervical dislocation The uterine horns ere removed and placed in Krebs-Ringer bicarbonate-solution bubbled continuously ith 95 O_2 and 5 CO_2. The composition of the Krebs-Ringer medium was (mM), 115 NaCl, 4 63 KCl, 0 1 $CaCl_2$, 1 16 $MgSO_4$, 1 16 NaH_2PO_4, 21 9 $NaHCO_3$ and 49 mM glucose.

Each horn was trimmed of excess fat and connective tissue cut open lengthwise and endometrium removed by scraping hile keeping the tissue in bubbling Krebs-Ringer solution throughout this period. The scraped myometrium as blotted dry and placed in ice-cold sucrose (0 25 M) and Hepes (10 mM) solution, pH 7.2. The tissue, after eighing, as homogenized in about 10 volumes of the above sucrose-Hepes solution ith Polytron homogenizer for 10 3 periods (per g tissue) ith intermittent pauses of 20 The homogenate was centrifuged at 1 000 g for 10 min and the pellet discarded. The supernatant was filtered through 3 layers of gauze and centrifuged at 15 000 g for 15 min. The resulting pellet, hich was enriched in mitochondria as occasionally saved for experiments dealing ith Ca uptake measurements in mitochondria. The above supernatant was centrifuged at 40 000 g for 1 h to obtain the microsomal pellet The pellet was suspended in sucrose Hepes solution to give protein concentration of 1 2 mg/ml and used for Ca uptake experiments immediately Protein concentration in the microsomal fractions was determined by the method of Lowry *et al.* (1951).

Measurement of Ca uptake

Microsomes were incubated at 37°C in 1 ml of solution containing (mM) 20 Hepes buffer (pH 7.25), 4 ATP 5 $MgCl_2$, 5 NaN_3 and 0 025 $CaCl_2$ including Ca^{45} The medium was supplemented with sucrose 0.2 M or KCl 0 1 M or NaCl 0.1 M. Microsomal protein concentration was 80–120 μg per ml. After incubation, 0 2 ml of the reaction mixture was filtered at various times under suction through Millipore filters (HAWP 04500). The filters were subsequently washed with 2 ml of Hepes buffer containing sucrose or KCl or NaCl depending on the composition of the reaction mixture. The radioactivity retained by the filters was counted by liquid scintillation spectrometry using Aquasol (New England Nuclear Corp.) as the scintillation fluid. Control tubes with the complete incubation mixture but without the microsomal protein were incubated in each assay Control filters bound less than 0 05 of the Ca^{45} present in the reaction mixture irrespective of its composition.

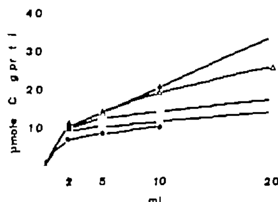


Fig. 1

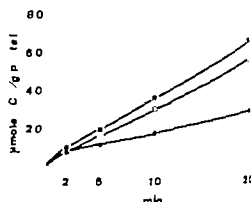


Fig. 2

Fig. 1 Ca uptake by rabbit myometrial microsomes in the presence of 2 mM Pi (■), 5 mM Pi (△), 2 mM oxalate (□), 5 mM oxalate (▲), and in the absence of potentuation anions (●). Uptake medium was supplemented with KCl.

Fig. 2. Ca uptake by rabbit myometrial microsomes from a medium supplemented with KCl (●) or sucrose (■). The middle curve represents data (□) of Ca uptake from a medi m supplemented with sucrose but microsomes after Millipore f ltration were washed with KCl see Methods.

Results

The effect of P_i on the time course of Ca uptake by rabbit myometrial microsomes is shown in Fig 1 Whereas 2 mM oxalate had no significant effect on Ca uptake, the same concentration of P_i increased Ca uptake slightly but significantly Both oxalate and P_i had little effect on the initial rate of uptake but a pronounced effect on the capacity of Ca uptake, measured after 20 min, and Ca uptake after this period was greater in the presence of 5 mM oxalate than that of 5 mM P_i When NaN was omitted from the medium, Ca uptake increased by only about 20% (not shown)

The presence of sucrose in the uptake medium had a significant effect on oxalate-induced potentiation of Ca uptake but not on that potentiated by P_i or on that measured in the absence of either potentiating anion (Table I) There was no significant difference in Ca uptake when the medium was not supplemented or was supplemented instead of sucrose with KCl or NaCl or choline chloride This was true in both the absence and presence of potentiating anions indicating that sucrose increased potentiation by oxalate rather than the cations depressed it.

Table I The effect of sucrose or electrolytes added as supplements to the reaction mixture on Ca uptake by rabbit myometrial microsomes. Ca ptak was determined after 20 min of incubation. Values are means ± S.E. of those btained f 4 or 5 separate e periments.

Supplement	Ca uptake (µmol/g prot)		
	without oxalate	with oxalate	with Pi
None	7.88 ± 1.0	23 15 ± 2.7	21.50 ± 2.4
Sucrose	10.66 ± 1 3	38.46 ± 4 8	26.76 ± 2.3
KCl	8.36 ± 1.2	20.39 ± 2.4	21 42 ± 2.0
NaCl	9.57 ± 0.9	19.96 ± 2.1	22.38 ± ..3
Choline chloride	12.88 ± 1.2	19.51 ± 1 7	26.54 ± 2.4

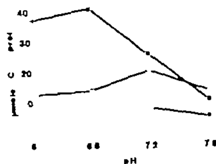


Fig 3

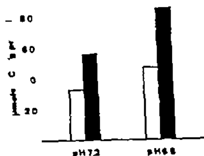


Fig 4

Fig. 3. Dependence on pH of Ca uptake in the absence of potentiating anion (●), or in the presence of P_i (□) or oxalate (■).

Fig 4. Effect of pH on oxalate-potentiated Ca uptake from medium supplemented with KCl (open bars) or sucrose (closed bars)

Microsomes that had accumulated Ca from a sucrose containing medium in the presence of oxalate lost some of the bound Ca when washed with KCl instead of sucrose solution (Fig 2), but the amount of Ca remaining in the microsomes was still considerably higher than that found after its accumulation from a KCl medium.

Another significant difference between the potentiating effect of oxalate and P_i was observed when the pH of the incubation mixture was varied (Fig. 3). In the absence of potentiating anions a pH change between 6.4 to 7.6 had little effect on Ca uptake. In the presence of P_i the maximum uptake was observed at pH 7.2 although the pH-optimum had relatively broad peak. When oxalate was used as a potentiator of Ca uptake, the pH-optimum was 6.8 but even at 6.4 the uptake was near maximum. However at pH 7.2 Ca uptake decreased by about 50% and was not significantly different than that observed with P_i at this pH. Increasing pH to 7.6 further reduced oxalate-supported Ca uptake.

Having observed that a reduction in pH or the inclusion of sucrose in the medium increased oxalate-supported, but not P_i-supported, Ca uptake considerably the effect of sucrose at lower pH (6.8) was examined. As seen in Fig. 4 lowering the pH had an additive effect to that observed by inclusion of sucrose in the medium. Thus, a reduction in pH from 7.2 to 6.8 along with the substitution of KCl by sucrose almost trippled the amount of Ca taken up

Discussion

The present results clearly demonstrate a potentiation by both oxalate and P_i of Ca uptake by myometrial microsomes. Rabbit myometrial microsomes prepared and assayed in the way as described here were also found to have much higher affinity for Ca uptake (Batra 1977) than that reported previously by us (Batra 1973 Batra 1975). Furthermore, a Ca-stimulated ATPase activity related stochiometrically to Ca uptake, could also be demonstrated (to be published)

The potentiating effect of P_i on Ca uptake by the microsomal fraction of smooth mus... was not unexpected but has not been demonstrated previously The present data clear... show that potentiation by P_i of Ca uptake differs not only quantitatively but also quali... tively from that observed with oxalate. Oxalate potentiation of Ca uptake in contrast to th... by P_i was sensitive to the composition of the medium as it was highly stimulated by t... presence of sucrose in the medium as well as by lowering of pH below neutrality The eff... of increasing H ion concentration and the presence of sucrose were additive. Whether t... stimulation is simply due to a greater permeability of oxalate to microsomal membra... under these conditions or whether it involves a more complicated mechanism cannot b... known In any event the present results showing a pronounced stimulation of Ca uptake b... sucrose or by a reduction in pH specifically in the presence of oxalate might provide a... explanation for some of the controversy on the oxalate potentiation of Ca uptake in smoo... muscle microsomes.

Our results on the pH influence on oxalate stimulated uptake are in agreement with the... recently published by Godfraind *et al* (1976) on intestinal smooth muscle microsom... Krall *et al* (1976) using a relatively alkaline (pH 8) reaction mixture recently reporte... oxalate potentiation of Ca uptake by rat myometrial microsomes. However the rates o... Ca uptake in their study were extremely low and uptake did not reach a plateau even afte... 30 min of reaction which is in contradiction with our previous data (Batra and Daniel 1971 a... as well as with those of Nishikori *et al* (1977) reported recently In fact, the data of Kral... *et al* showed practically no ATP-dependent uptake in the first 5 min whereas the data o... other two studies mentioned above (Batra and Daniel 1971 a, Nishikori *et al* 1977) showed... that Ca uptake reached a plateau after 5 min. In view of this it seems difficult to correlat... the kinetics of Ca uptake presented by Krall *et al* with the physiological function. N... explanation was given by the authors for either these extremely slow rates of Ca uptake... the use of a reaction medium at pH 8

The present results also showed that a part of the Ca taken up under the influence of sucrose in the presence of oxalate could be released by KCl, but not by sucrose, washing This would indicate some Ca binding at the surface of the vesicles for which K can compete with Ca^{++} However there is a fraction of Ca which is probably transported inside the vesicle under the influence of sucrose as KCl washing removed only 39% of additionally accumulated Ca. The study of the mechanism of this action of sucrose deserves further attention. Interestingly sucrose has been shown to antagonize the inhibition by certain drugs and elevated temperature of Ca uptake by cardiac sarcoplasmic reticulum (Solaro *et al.* 1972). Since not only the effect of pH but also of sucrose was specific for oxalate, it is suggested that Ca is taken up by a different mechanism in the presence of oxalate than in its absence or when oxalate anion is substituted with inorganic phosphate.

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Acta physiol. scand. 1978 104 74–81

From the Department of Cell Biology University of Jyväskylä, Finland

Oxidative and lysosomal capacity in skeletal muscle of mice after endurance training of different intensities

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Abstract

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The activity of certain enzymes of energy metabolism (cytochrome c oxidase, citrate synthase, mal[illegible] dehydrogenase and lactate dehydrogenase) and of lysosomes (β-glucuronidase, β-N-acetylglucosaminida[illegible] arylsulphatase, ribonuclease, deoxyribonuclease acid phosphatase and cathepsin D) was assayed in *m. rectus femoris* of mice trained 5 days per week, 1 h per day f r 4 weeks according to 4 different program[illegible] mes. I. running speed 20 m/min, horizontal track II. 25 m/min, horizontal track, III. 20 m/min 8° uph[illegible] inclination, and IV 25 m/min 8 uphill inclination. Oxidative capacity increased and anaerobic capa[illegible] decreased without distinction between the different training programmes. Of acid hydrolases assay[illegible] the activities of β-glucuronidase and cathepsin D were increased independently of training intens[illegible] Simultaneous histochemical observations on β-glucuronidase and arylsulphatase activities in [illegible] contralateral *m. rectus femoris* showed more intense staining in red as compared to white muscle fib[illegible] It is suggested that training affected the red fibres and that the applied level of loading was probably [illegible] low to cause major involvement of whit f bres.

K y ords. Acid hydrolases, skeletal muscle endurance training, training intensity oxidati capac[illegible] mouse

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The type, duration and intensity of exercise as well as the fibre type profile of musc[illegible] involved in performance are generally accepted as factors of major importance influenci[illegible] the magnitude and nature of responses occurring in the exercised muscles. Recent[illegible] attention has been paid to the quantitative aspects of these factors in endurance exerci[illegible] (Fitts *et al* 1975, Terjung 1976, Dohm *et al* 1977). In these investigations the activities [illegible] energy metabolism enzymes have been under the study

The activities of some lysosomal hydrolases are also affected by physical exercise. The activity of β-glucuronidase is increased in crude skeletal muscle homogenates of endurance-trained mice (Vihko *et al.* 1974, Pilström *et al.* 1978). The following investigation was aimed to study the effects of different training intensities on the activity and localization of certain acid hydrolases and on activity of certain enzymes of energy metabolism.

Material and methods

Animals and training

All 83 male NMRI mice, aged 70–80 days at the beginning of training, were used in the experiments. Before training the mice were randomly divided into 5 groups. I. Untrained controls (n = 22) living under normal cage conditions, II. A group (TR 20/H) (n = 15) running on a horizontal track of a motor driven treadmill at a speed of 20 m/min for 1 h per day. III. A group (TR 25/H) (n = 14) running on a horizontal track at a speed of 25 m/min for 1 h per day. IV. A group (TR 20/U) (n = 16) running uphill (inclination 8°) at a speed of 20 m/min for 1 h per day, and V. A group (TR 25/U) (n = 16) running uphill (inclination 8°) at a speed of 25 m/min for 1 h per day. The animals were exercised 5 days a week. During the first 8 days of training the running time was increased from 30 to 60 min and speed from 12 to 20 or 25 m/min simultaneously and equally for each group until the desired speed was obtained. The exercise remained at this level 16–18 days and was terminated the day before the mice were killed. Two motor driven treadmills, one with horizontal and the other with an uphill track were used in the training. The animals were exercised at fixed times (10–11 a.m. groups TR 20/H and TR 20/U, 11.10–12.10 a.m. groups TR 25/H and TR 25/U) each day. Day in the cages was artificially divided into 12 h of light and 12 h of darkness changing at 6.00 p.m. The mice were housed 7 or 8 mice to a cage (Type IV, Scanbur, Denmark), and they had free access to solid food pellets (Astra-Ewos R 3, Sweden) and tap water. The normally growing control mice, feeding *ad lib.*, gained more weight during the study (Table I) as compared to all the trained groups. This confirms earlier observations made with rats (Terjung 1976). The temperature (21–22°C) and humidity (40%) were automatically kept constant in the animal house.

Tissue preparation and assay methods

Animals were killed by dislocation of the neck. Skin was removed and *m. quadriceps femoris* dissected and further divided into separate muscles of which *m. rectus femoris* was studied. The muscle was immediately weighed. No differences in the weights between trained and control groups were observed (Table I). The sample was homogenized in ice-cold homogenization medium (150 mM KCl, 50 mM $KHCO_3$ and 6 mM EDTA, pH 7.4) in a Potter-Elvehjem homogenizer with glass pestle. The homogenates (3 w/v %) were kept at −18°C until analyzed within two weeks. Before assaying the activity of lysosomal enzymes an aliquot of the preparate was made 0.1% in respect to Triton X-100 concentration in order to determine total activities of lysosomal hydrolases.

Activities of the following acid hydrolases were assayed: β-glucuronidase (β-GUase) (EC 3.2.1.31), β-N-acetylglucosaminidase (β-GAase) (EC 3.2.1.30), p-nitrophenylphosphatase ("acid phosphatase") (p-NPPase) (EC 3.2.3.2), cathepsin D (Cat. D) (EC 3.4.4.23), arylsulphatase (ASase) (EC 3.1.6.1), ribonuclease (RNase) (EC 2.7.7.16), and deoxyribonuclease (DNase) (EC 3.1.4.6). Acid hydrolase assays were performed essentially as described by Barrett (1972).

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Because the biochemical activity of acid hydrolases in skeletal muscle is partly derived from muscle cells and partly from non-muscle cells such as macrophages and connective tissue cells (e.g. Canonico and

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The type, duration and intensity of exercise as well as the fibre type profile of muscles involved in performance are generally accepted as factors of major importance influencing the magnitude and nature of responses occurring in the exercised muscles. Recently attention has been paid to the quantitative aspects of these factors in endurance exercise (Fitts *et al.* 1975 Terjung 1976, Dohm *et al* 1977) In these investigations the activities of energy metabolism enzymes have been under the study

TABLE II. The activities of cytochrome oxidase (CytOx), citrate synthase (CitSy), malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) in the thigh muscle (*m. rectus femoris*) of four groups of mice trained according to different training programmes. Activities are expressed as nmol substrate consumed/min/mg muscle wet weight at 25 C, except for CytOx at 28 C. Statistical significance as in Table I.

Group		CytOx	CitSy	MDH	LDH
Controls	22	14 09 ± 2.47	31 77 _ 5.21	255.1 ± 34 4	366.7 ± 61.2
I Horizontal, 20 m/min	15	18 68 ± 2.48	38 92 ± 5 81	314 8 ± 40.7	340 0 _ 67 7
II Horizontal, 25 m/min	14	18 20 ± 3 16	39 01 ± 6.74	329 7 ± 50 4	324 0 _ 68 4
III Uphill, 20 m/min	16	19 15 ± 3 25	40 55 ± 4 21	331 9 ± 42 9*	337 1 ± 55 4
IV Uphill, 25 m/min	16	19.24 ± 2 52	41.20 ± 5 73	348 8 ± 47.5	333 4 _ 60 6
I–IV All trained	61	18 84 ± 2 81	39 97 ± 5 60	331 6 ± 45 4	333.8 ± 61.2
I–II All horizontal	29	18 45 ± 2.74	38.96 ± 6 05	332 0 ± 44.7	332.3 ± 66.1
III–IV All uphill	32	19 20 ± 2 82	40.88 ± 4 88	340 4 ± 44 2	335.3 ± 56 2

Histological and histochemical observations

Glucuronidase activity was more prominent in red muscle fibres, especially in their perinuclear area, as compared to white fibres in which only minor activity was sometimes seen in interfibrillar space (Fig. 1 B). Activity granules appeared longitudinally arranged between myofibrils as also in the case of arylsulphatase activity (Fig. 1 C). β-Glucuronidase activity was only occasionally seen outside muscle cells. Arylsulphatase staining appeared only in red fibres and some cells (probably fibroblasts) seen between fibres (Fig. 1 C D). In histological samples some degenerative and regenerative fibres were seen both in control and in trained animals.

Histochemical and histological examination did not reveal any major quantitative or qualitative differences between the trained (TR 25/U) and control groups.

Discussion

In the present study the duration of daily exercise was set to 1 h due to previous experience with mice, which showed that moderate 1 or 2 h daily running exercise caused a similar

TABLE III. The activities of β-glucuronidase (β-GUase), β-acetylglucosaminidase (β-GAase), arylsulphatase (ASase), p-nitrophenylphosphatase (p-NPPase), ribonuclease (RNase), deoxyribonuclease (DNase) and cathepsin D (Cat.D), in the thigh muscle (*m. rectus femoris*) of four groups of mice trained according to different training programs. Activities are expressed as pmol/min/mg muscle wet weight at 37°C. Statistical significance as in Table I

Group		β-GUase	β-GAase	ASase	RNase	DNase	Cat.D	p-NPPase
Control	22	6 74 ± 0 77	235 ± 53	29 3 ± 5.2	301 ± 30	190 ± 20	83 4 ± 24.9	1 242 ± 78
I Horizontal, 20 m/min	15	7.96 ± 1.11 **	239 ± 43	25.0 ± 2 9	286 ± 53	193 ± 18	99.3 ± 26 4	1 241 ± 91
II Horizontal, 25 m/min	14	8 98 ± 0 77*	246 ± 51	28.1 ± 2 3	320 ± 48	199 ± 16	95 4 ± 20 4	1 291 ± 95
III Uphill, 20 m/min	16	7.86 ± 0 99	240 ± 52	29 6 ± 4.7	319 ± 42	200 ± 17	97.9 ± 30 2	1 266 ± 100
IV Uphill, 25 m/min	16	9 11 ± 4.53	238 ± 65	27.5 ± 5 8	304 ± 53	191 ± 28	94 1 ± 25 9	1 238 ± 92
I–IV All trained	61	8.47 ± 2 45	243 ± 52	27 6 ± 4.4	307 ± 49	196 ± 20	96.7 ± 25 3	1 258 ± 94

TABLE I Weights of experimental animals and their *rectus femoris* -muscle together with protein of the *rectus f moris*-muscle in control group and four d fferent trained groups. Mean + sr deviation The significance of differences. $p<0.05$, $p<0.01$ and $p<0.001$

Group	n	Weight of lmals (g)	Weight of the rectus-muscle (mg)	Protein content of the rectus-muscle (µg mg)
I Controls	22	39.5±3 6	107.3±14.3	179.3±16 0
II Horizontal, 20 m/mi	15	35 6±2.5	103 1±14.6	182.7±12.0
III Horizontal, 25 m/min	14	37 0±3 0	110.3±10.3	180.7±9.3
IV Uphill, 20 m/min	16	37 4±2.4	110 0±8.5	180.7±15 0
V Uphill, 25 m/min	16	36.5±2.7*	105 0±10.5	180 0±11.3

Bird 1970), 2 representative acid hydrolases (β-GUase and ASase) were stained histochemically in cryostat sections. *M rectus femoris* from the contralateral leg to the part used for biochemical assays removed, cut i to two parts which were embedded on specimen block with OCT-compound (Tissue Tech, Ames) for longitudinal nd cross sectioning. Samples were freezed in isopentane prechilled by liquid nitrogen. Serial cryostate sections for the staini g of NADH-diaphorase (Novikoff *et al.* 1961), β-glucuronidase (Chayen *et al* 1973), and ryIsulphatase (Goldfisher 1972) activities and for hematoxylin-eosin staining were cut with Ames Cryostate II and mounted on cover glasses. On the basis of NADH-diaphorase activity individual muscle cells were classified either as red or white f bres, *i.e.* having high or low oxidative capacity respectively Histochemical analysis was performed only on control and TR 25/U groups.

St tistical methods

Standard procedures were used to calculate means and standard deviations (SD). The statistical significance of differences between the means of control and the trained groups were tested by Student's t-test for non-correlating means.

Results

Enzymes of energy metabolism

The activities of all estimates of aerobic metabolism (CytOx, CitSy and MDH) were statistically significantly increased due to the different training programmes (Table II). This increase in oxidative capacity was in the mean of 30%. On the contrary LDH activity slightly decreased, the decrease being significant only when all values of trained animals were pooled Depending on work intensity slightly higher increases in oxidative capacity were observed after uphill training than after horizontal training. This effect, like the effect of running speed, was not statistically significant

Acid hydrolases

Training increased β-GUase activity in all exercised groups regardless of intensity (Table III) Systematically higher values in trained groups were found in Cat D activity the increase was significant ($p<0.05$) only for the pooled values of trained animals. In other activities (β-GAase, RNase, DNase, and p-NPPase) no significant differences were observed between control and trained animals. ASase activity decreased in group II (TR 20/H).

The protein concentrations of *m. rectus femoris* were similar in all animal groups (Table I)

ilable data on anaerobic or lysosomal capacity do not permit similar conclusions as to influence of daily exercise time.

Benzi *et al.* (1975) suggest that the magnitude of the adaptation in oxidative capacity (succinate dehydrogenase, cytochrome oxidase and NADH cytochrome c reductase activities) rat gastrocnemius and soleus muscles is dependent both on daily work load and on total training time. Daily work load was varied both in respect to the duration and speed of running. The design of their study does not permit evaluation of the effects of speed or running-time separately. In a study by Dohm *et al.* (1977) with rat gastrocnemius the variations in daily running speed during a steady-speed endurance training programme of weeks did not significantly affect the activity of succinate dehydrogenase, and the results of Terjung (1976) show slight and insignificant increases in cytochrome c concentration and citrate synthase activity in rat gastrocnemius as a result of raising the elevation of the treadmill.

In agreement with Dohm *et al.* (1977) and Terjung (1976) the present results (CytOx, MDH, and CitSy) show slight but insignificant effects of speed or higher intensity caused by treadmill elevation on the oxidative capacity of the rectus femoris-muscle of mice. Neither the training changes in anaerobic (LDH activity) or lysosomal capacity (Table III) significantly affected by the intensity of training.

Adaptation of the oxidative capacity of mixed skeletal muscle to moderate endurance training seems thus be more affected by the length of the daily exercise period (Fitts *et al.* 1975, Dohm *et al.* 1977), if the duration is below a hypothetical threshold value (approximately 2 h for the rat, 1 h or less for the mouse), than by relatively slight changes in intensity (speed or extra load caused by treadmill elevation). If, however both duration and speed are increased simultaneously the response in oxidative capacity is stronger (Benzi *et al.* 1975).

The muscle under study (*m. rectus femoris*) is a mixed skeletal muscle, therefore our results represent the average of all heterogeneous adaptive responses, which have occurred in the three, metabolically different, fibre types. Moderate endurance training programmes result in an increase in the oxidative or respiratory capacity of all three (slow-twitch red, fast-twitch red, and fast-twitch white) skeletal muscle fibre types in rat (Baldwin *et al.* 1972) and similarly Salminen *et al.* (1977) showed an increase both in predominantly red and predominantly white parts of the quadriceps femoris-muscle of mice. Increases in oxidative capacity after moderate endurance training have always been more pronounced, in chemical units, in red as compared to white muscle tissue, although relatively they may be almost equal in the two tissue types both in the rat and the mouse (Baldwin *et al.* 1972, Salminen *et al.* 1977). There seems also to be a tendency for a higher increase of β-glucuronidase activity in red muscle tissue compared to white in mice as a response to moderate endurance exercise programme (Vihko *et al.* 1977 a).

Glycogen depletion studies by Armstrong *et al.* (1974) with rats showed that contractile activity at low work intensities is maintained by oxidative fibres (both slow and fast twitch), and that major use of anaerobic fibres (fast-twitch glycolytic type) occurs at high work intensity (increased running speed) or after depletion of glycogen stores in oxidative fibres. In contrast to gastrocnemius muscle (mixed) Terjung (1976) observed an effect of

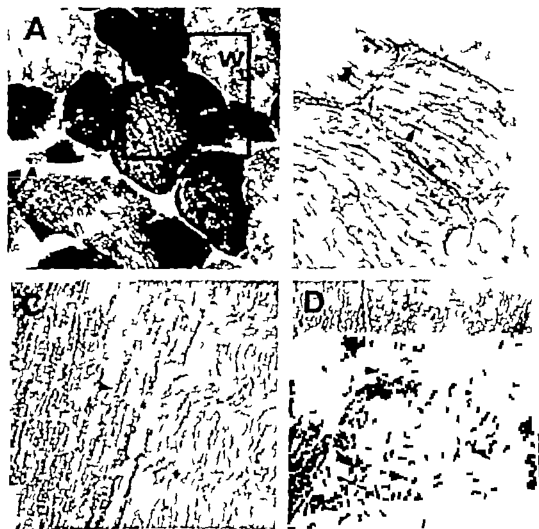


Fig. 1 *A*. NADH tetrazolium reductase ("diaphorase") staining showing the classification of fibres: highly oxidative red (darkly stained) and low oxidative white fibres (moderately or slightly stained). Section from control animal. Magnification × 290. *B*. Serial section for β-glucuronidase staining from control animal, outlined area from Fig. 1 A. Slight activity in control samples was usually seen in red fibres as in this case (arrows) but usually not in white fibres (w). Occasional activity was now and then seen outside muscle fibres. Magnification × 675. *C*. An example of arylsulphatase staining, trained animal. Activity is typically seen only in red, highly oxidative fibres (left). Magnification × 875. *D*. Arylsulphatase staining from control animal. In this case some activity is also seen in structures outside muscle fibres in addition to typical activity in red, highly oxidative fibres. Magnification × 625

response in the activity of MDH or β-GUase (Vihko *et al.* 1974). With rats Fitts *et al.* (1975) reported an increase in oxidative capacity (cytochrome c concentration, citrate synthase activity and malate-puryvate oxidation rate) up to 2 h daily running exercise and Terjung (1976) showed that running for longer periods than 2 h per day did not further increase the oxidative capacity (cytochrome c concentration and citrate synthase activity) of rat gastrocnemius. Thus, at least with rodents, the influence of the duration of daily exercise on the oxidative capacity of the working "mixed" muscles during moderate endurance training at a steady speed is such that maximal adaptative response is achieved after a certain threshold time and further exercise of equal intensity is not effective

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higher treadmill incline on markers of oxidative capacity in fast twitch white (white m fibres indicating an increased involvement of white muscle with increasing work l The intensity factors (speed and higher running incline) thus especially affect white m

The training effects of the present experiment on oxidative capacity in mouse m femoris-muscle were evidently directed to red fibres, and the upper range of intensity (es especially running speed was too low to cause continuous recruitment of white muscle fi

The physiological significance of increased β-glucuronidase or cathepsin D activity i moderate endurance training is not clearly understood The rest level activity of these i some other acid hydrolases is higher in predominantly red as compared to white skel muscle tissue in mice (Vihko *et al* 1978 a) Moderate endurance training or one exhaus exercise bout also affects β-glucuronidase activity more in red than in white m (Vihko *et al* 1978 a, b) The general concept of the functions of lysosomal hydrolases (Duve 1973) suggests that increased activities parallel increased catabolism.

Increased activity of β-glucuronidase might be a reflection of a changed catabolic lev the homeostasis of endurance trained muscle. Cathepsin D activity correlates with leu [14]C incorporation rate in human skeletal muscle (Lundholm and Schersten 1975) E hanced anabolism, which in trained muscle is obvious on the basis of *e.g.* increased m chondrial capacity might be compensated by increased lysosomal activity Histochem observations reported here show that biochemically found increases in β-glucuronid activity probably occur inside muscle fibres and not *e.g.* in connective tissue cells as have previously suggested (Vihko *et al* 1974, Pilström *et al* 1978). The training indu increase in β-glucuronidase activity seems to be more pronounced in red, oxidative mus fibres (Vihko *et al* 1978 a) The involvement of red muscle cells during moderate, prolong endurance training probably is thus also demonstrated by the activity of β-glucuronida

The hypothesis of increased lysosomal capacity in connection with increased contract activity of skeletal muscle was supported by the activities of β-GUase and Cat.D ot whereas the activities of β-GA-ase RNase DNase and p-NPPase were unchanged r ASase activity even decreased after the lightest training programme Any definit explanation for the differentiation of training-induced adaptations (Table III) in enzy activities, which usually function as an entity (de Duve 1973) cannot be given, although t differentiation may simply reflect the strictly directed effects of endurance training ab in the catabolic capacity of muscle fibres.

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The mechanisms underlying these specific effects on the action potential and the ceptor potential have been discussed in terms of detergent-membrane interactions ydqvist 1977). Both detergent-lipid and detergent-protein interactions seem to be in- lved. Although experimental findings favour the idea of a detergent-protein effect, at least r the decrease in the fast sodium current in nerve and muscle, it has not been possible to mpletely rule out either alternative.

The frog neuromuscular junction, where acetylcholine binds to the nicotinic acetylcholine ceptor protein, thereby inducing a depolarization in the postsynaptic membrane, is well aracterized both electrophysiologically (Katz 1966) and morphologically (Birks *et al.* 1960, outeaux *et al.* 1970, Dreyer *et al.* 1973, Heuser *et al.* 1974). Furthermore, receptor roteins from electric organs have been purified and characterized biochemically (see hangeux *et al.* 1975 Heilbronn 1976). This makes the end-plate a suitable preparation for harmacological-toxicological studies and in particular for studies of effects on the ostsynaptic cholinergic processes in isolation by iontophoretic application of acetylcholine. he present study was undertaken in order to further examine the action of Triton deter ents. The action of Triton X-45 and Triton X 100 on different processes involved in euromuscular transmission was investigated. In particular the effect on the postsynaptic embrane, *i.e.* the acetylcholine receptor was studied. Electrophysiological measurements ere combined with ultrastructural observations of the muscle end-plate, in order to stablish if morphological changes were produced by Triton detergents.

Methods

The experiments were performed on isolated cutaneous pectoris muscles of the frog (*Rana temporaria*). This muscle was chosen because it is very thin (only about three layers of muscle fibres) and consequently influence of the detergent to the end-plate region should be optimal (cf. Dreyer *et al.* 1974). The muscles were dissected free and placed with its deep surface upward in a perspex chamber (volume 2 ml). The normal Ringer solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 2.15 mM Na_2HPO_4 and 0.85 mM NaH_2PO_4, pH being 7.0–7.2 (Adrian 1956). All experiments including the fixation procedure were performed at room temperature (22°–24°C).

Electrophysiological recordings. The experimental procedures were in part the same as described earlier (Rydqvist 1977). Intracellular membrane potential changes were recorded with glass capillary micropipettes filled with 3 M KCl, resistance about 10 MΩ and tip potential less than 5 mV. Micropipettes used to inject current were filled with 2 M K-citrate, resistance 15–20 MΩ. The micropipette used to apply acetylcholine iontophoretically was filled with 2 M acetylcholinechloride (Sigma) the resistance being 30–40 MΩ.

The recording micropipette was connected to a high input impedance amplifier (× 10). The output from this amplifier was fed into an oscilloscope (Tektronix 5103 N), a digital volt-meter (to display resting membrane potential) and an amplifier (Tektronix Am 502) which could be either DC (0–3 000 Hz) or AC-coupled (0.1–3 000 Hz). The output from this amplifier was usually set so that a total amplification of 200 times was achieved and was used to record spontaneous miniature end-plate potentials (m.e.p.p.'s) or iontophoretically induced end-plate potentials. The × 10 and × 200 outputs were in addition fed into two channels of a tape recorder (Hewlett and Packard 3960). The recording speed was 3 3/4 inch/s which is equivalent to a bandwidth of 0–1 250 Hz. The membrane potential changes thus stored on tape could later be displayed on the oscilloscope and recorded on photographic film. The current passed through the muscle fibre or the iontophoretic micropipette (see below) could be monitored on the oscilloscope as the voltage drop across the feedback resistor in an operational amplifier circuit used to hold the bath at virtual ground potential. This signal was also fed into one channel of the tape recorder via a × 250 DC amplifier.

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Triton detergents and the frog neuromuscular end-plate An electrophysiological and ultrastructural study

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Abstract

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The effects of the nonionic detergents Triton X-45 and Triton X 100 were studied in the frog muscle end plate by intracellular recordings of spontaneous miniature end-plate potentials (m.e.p.p. s) and the potential changes produced by iontophoretic application of acetylcholine (ACh-potentials). In addition, the ultrastructural changes produced by Triton X 100 were studied by transmission electron microscopic and freeze-fracture techniques. It was found that Triton X-45 and Triton X 100 caused a rapidly developing reduction of the amplitude of the m.e.p.p. s. The response to iontophoretic application of acetylcholine was reduced by Triton X 100. Following return to normal Ringer solution the ACh-potentials recovered although not completely The dissociation constant calculated from the rate constants for onset and offset of the reaction ($K_D = k_2/k_1$) was 5–50 μM depending on the type of stoichiometric reaction presumed to occur between Triton X 100 and the cholinergic receptor The ultrastructural changes observed indicate that the nerve terminal plasma membrane and mitochondria are affected by Triton X 100. Leakage of Ca^{2+} from the latter may therefore be the cause of the increase in m e p.p. frequency It is concluded that the influence on the amplitude of the m.e.p.p. s and the ACh-potentials can be attributed to a direct effect of the detergent upon the acetylcholine receptor protein.

Detergents of the nonionic Triton series, in particular Triton X-45 and Triton X 100 have been shown to have specific effects on the excitability of nerves and muscle. The action potential in frog skeletal muscle fibre is suppressed the maximum rate of rise of the impulse being reduced to 50% by Triton X 100 (40 10^{-6} M) (Rydqvist 1977) These results and work on frog myelinated nerve fibres (Brismar and Rydqvist 1978) have provided evidence that the suppression of the action potential is most likely due to an effect on the fast initial increase in sodium permeability which underlies the action potential in nerve and muscle (Hodgkin *et al* 1952, Adrian *et al* 1970) It has also been demonstrated that Triton detergents block the action potential in crustacean stretch receptor neurons and that prolonged exposure reduces and finally abolishes the receptor current. This block of the transducer action of the neuron may be attributed to a specific action on the sodium permeability increase during stretch as revealed by voltage clamp analysis (Ottoson and Rydqvist 1978).

The mechanisms underlying these specific effects on the action potential and the receptor potential have been discussed in terms of detergent-membrane interactions (Rydqvist 1977). Both detergent-lipid and detergent-protein interactions seem to be involved. Although experimental findings favour the idea of a detergent-protein effect, at least for the decrease in the fast sodium current in nerve and muscle, it has not been possible to completely rule out either alternative.

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Acetylcholine was ejected by applying positive voltage pulses to the pipette. In some experiments a small

negative (usually less than 1 volt) DC potential was applied to the inside of the pipette in order to inhib diffusion of acetylcholine out of the pipette. The positive pulses used to release ACh had an amplitude 1 to 6 V with a duration of 4 ms. The current produced by the applied voltage ranged between 0.3–2 A and could be monitored by the voltage current converter described above. The duration and the current used are equivalent to a charge of $0.1–1.0 \times 10^{-9}$ Coulomb. The current pulses were usually not display during the experiment. The depolarization response due to release of ACh from the micropipette is refer to as "ACh potentials" (del Castillo and Katz 1955).

A superficial end-plate was located with the aid of a 100 stereo microscope fitted with dark field illumination and the recording microelectrode inserted into the fibre. If the resting membrane potential was stable for about 5 min and the miniature e.p.p. of moderate amplitude the iontophoretic pipette was carefully approached to the same region and placed within 100 μm from the recording microelectrode. With small relocations it was usually possible to find a spot where ACh-potentials could be released by repetitive pulses delivered every 14 s. This interval betwee pulses was found not to desensitize the end-plate (Thesleff 1955, Katz *et al.* 1956). If the ACh-potentials and the recording conditions in general remained stable for about 30 min the actual experiment was started. In many fibres either the resting membrane potential deteriorated or the ACh potential was not stable. In these cases the fibres were rejected. The number of fibres which were used to follow effects of long-time exposure of Triton detergents were limited because small fibres which are known to have large m.e.p.p.'s sometimes depolarized after relatively short periods of time following insertion of the microelectrode. On the other hand, fibres which were large judged from the small m.e.p.p.'s were more difficult to use for accurate measurements of both m.e.p.p. amplitude and frequency.

Application of substances was performed by adding about ml of the test solution and a subsequent withdrawal of the same volume. This was made three times for each substance to be tested. In this the solution in the bath could be changed to about 90% in about 30 s, without dislodgement of the electrodes. In particular the iontophoretic electrode was very sensitive to fluid movements in the bath and even with carefully performed changes of solution this pipette was sometimes moved in relation to the muscle fibre as judged from the change in amplitude of the ACh-potentials. Such fibres were only used to follow the change in miniature end-plate potentials (m.e.p.p.s) provided the recording electrode was still inside the cell.

Electron microscopy. Cutaneous pectoris muscles were carefully dissected free and pinned down at resting length in two identical chambers. One muscle was treated with the detergent solution (Triton X 100 160 10^{-6} M) the other kept as a control. Muscles with visibly damaged fibres were not used. All further procedures were performed at room temperature (+22 – +24°C). After treatment with the detergent usually for 30 min, both muscles were fixed for 60–90 min. The fixative used was either 2.5% glutaraldehyde and 2% paraformaldehyde or 3% glutaraldehyde and 5% paraformaldehyde in Na-cacodylate buffer at pH 7.1–7.3 to which 2 mM $CaCl_2$ and 2 mM $MgCl_2$ was added. Buffer solutions of concentration of 0.05 to 0.10 M were used. After fixation small pieces (about 0.5 mm) rich in end-plates were cut out from the muscle and postfixed for 60 min in 2% OsO_4 in the corresponding buffer. After rinsing in buffer the specimens were mordanted with 1% tannic acid according to Simionescu and Simionescu (1976). After dehydration in alcohol and embedding in Epon, ultrathin sections were cut on an LKB-ultrotome, stained with uranylacetate (Watson 1958) and lead citrate (Reynolds 1963) and examined in a Zeiss EM electron microscope. All solutions used were freshly prepared except for the OsO_4 solution which was used within one week after preparation. To obtain freeze-fractured replicas the same primary aldehyde fixation were used. End-plate rich regions (about 0.5 mm) were cut out and equilibrated in 30% glycerol for 30 min at room temperature. The tissue was frozen in Freon 22 (monochlorodifluormethane) cooled to −146°C by liquid nitrogen, fractured at −100°C and replicated with a 60 etching in Balzers 360 M apparatus fitted with an electron beam gun for Pt/C shadowing and a quartz crystal monitor to provide standardized replica thickness of about 2.0 nm. Replicas were cleaned in Na-hypochlorite H_2O–H_2SO_4–H_2O and mounted on formvar coated copper grids. Illustrations are positive images where regions of platinum deposits are dark while absence of platinum appears light.

Triton X-45 and Triton X 100 were gifts from Rohm & Haas Co (Sweden) and used without further purification. These detergents belong to a series of homologues with the general formula $C_nH_{2n+1}C_6H_4O$-$(CH_2CH_2O)_mH$ (Triton surface active agents. The nonionic octylphenoxy-ethanol (OPE) series. CS-40 I/ d, Rohm & Haas Co, Philadelphia). *m* is 8 and Triton X-45 (M.W. 426) has mean n-value of 4.5 and Trition X 100 (M.W. 628) has a mean n-value of 9.8 (Handbook of Physical Properties, CS-Ole Rohm & Haas Co, Philadelphia, Simons et al. 1973). The hydrophilic lipophilic balance number (HLB) is 10.4 for Triton X-45 and 13.5 for Triton X 100.

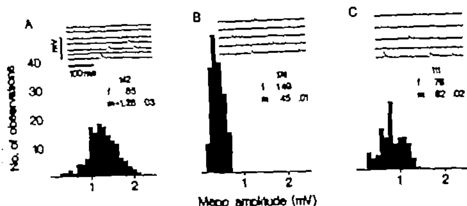


Fig 1 Effect of Triton X 100 (160 10^{-4} M) on the amplitude and frequency of m p p. Histograms from distribution of m p p. amplitude before (A) and after 13–15 min in Triton X-100 (B). At about 15 min the fibre as returned to normal Ringer solution and after an additional 30 min m e.p p. amplitudes ere recorded (C) Inserts show representative traces of m p p.'s. m mean ± S E. of m p p amplitude in mV mean m p p. frequency in s^{-1} number of m p p 's. Resting membrane potential: A 85 mV B 83 mV C −87 mV

Results

The effects of Triton detergents on the amplitude and frequency of the spontaneous m e.p.p. s ere first studied. Membrane potential and input resistance (R_i) were also followed. In a series of experiments iontophoretic application of acetylcholine was performed and change in response studied as a result of Triton X 100 treatment. The time course of onset and offset of the reaction between the detergent and the end-plate was used to calculate the dissociation constant (K_D). Prostigmin (10^{-4} g/ml, 3 3 10^{-4} M) was used in some experiments but no effect on the action of the detergent was observed.

Effect on spontaneous miniature end-plate potentials (m.e p p). A series of experiments was carried out to study the effect of Triton X 100 and Triton X-45 at different concentrations on the miniature end-plate potentials (m e.p p 's). It was found that both Triton X-45 and Triton X-100 in concentrations of 80–160 10^{-4} M (*cf* Rydqvist 1977) reduced the amplitude of the m e.p.p.'s within 5–30 min, the time course varying with concentration and also from preparation t preparation. In general 160 10^{-4} M of both detergents invariably abolished the m e p.p. s after about 15 min. The difference in effect between muscle fibres is most likely due to variations in the diffusion barriers at the end-plate, g some of the end-plates studied might have been located on the inferior side of the fibre. At lower concentrations, *e.g* 40 10^{-4} M, the time required to obtain clear effects was considerable, and these concentrations were therefore not used in the present study In Fig. 1 is shown the distribution of amplitude and the frequencies before, during and after application of Triton X-100 (160 10^{-4} M). This fibre was chosen to illustrate typical detergent effects because the m.e p p. s were not completely abolished and it was thus possible to construct amplitude histograms and to calculate mean m.e.p.p. frequencies. As seen the amplitude is reduced to about one third of the control amplitude after 13–15 min. After return to normal Ringer solution (at 15 min) the amplitude increased but did not

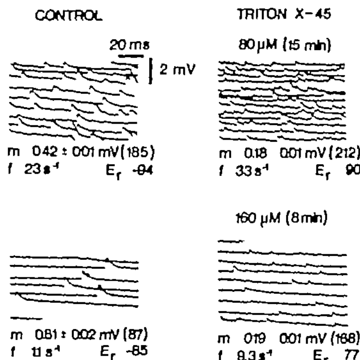
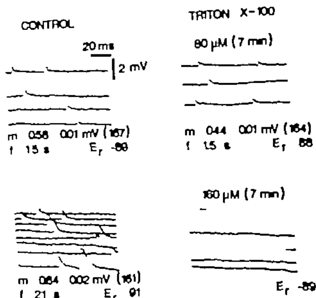


Fig. — Records of m.e.p.p.'s from two fibres showing the effect of Triton X-45 at two concentrations (80 and 160 10^{-6} M). From the recordings in normal Ringer solution (control) and in detergent solution the mean ±S.E. (m) and the frequency (f) of the m.e.p.p. are calculated using additional traces not shown in the picture. Numbers in brackets indicate number of m.e.p.p.'s used for these calculations. The resting membrane potential (E_r) in mV. Prostigmine 10^{-6} g/ml.

return to control value. As shown in earlier work on frog muscle action potential (Rydqvist 1977) the time needed to complete recovery is considerable. It is probable that this may partly be ascribed to deterioration of the muscle fibre or to factors which are not dependent on the detergent treatment *per se*.

The frequency of the m.e.p.p.'s was seen to increase from a control value of 0.8 s^{-1} to 1.5 s^{-1} after about 15 min in Triton X 100. After return to normal Ringer solution the frequency decreased and attained a value of 0.8 s^{-1} identical to that of the control. The increase in frequency was seen in most fibres treated with both Triton X-45 and Triton X 100 as is illustrated in Fig. 2 and Fig. 3. These figures show the effect of Triton X-45 and Triton X 100 respectively on the amplitude and frequency of the spontaneous activity of different end-plates. The increase in frequency due to the detergents is best illustrated at 160×10^{-6} M Triton X-45 (Fig. 2) while in Fig. 3 there is no increase at 80 10^{-6} M and at 160×10^{-6} M the m.e.p.p.'s are almost completely abolished so that an estimate of the frequency is impossible. As is also evident in Fig. 2 and Fig. 3 there is no basic difference between the effect of Triton X-45 and Triton X 100 which is in accordance with earlier work on frog muscle and on the crustacean stretch receptor (Rydqvist 1977, Ottoson and Rydqvist 1978). As the variability of the frequency increase was considerable the effect of Triton on the frequency of the spontaneous activity was studied on five fibres treated with Triton X 100, 160 10^{-6} M. End-plates were chosen for which the spontaneous m.e.p.p.'s were only partially suppressed. The results are outlined in Table I and it can be seen that there is a consistent



ig 1. Records of m.e.p.p.'s om two fibres showing the effect f Triton X-100 at tw concentrations (80 and 160 10^{-6} M). and f as in Fig. 2 Numbers brackets indicate number of p.p.'s used. The resting membrane potential (E_r) in mV rontogemme 10^{-6} g/ml.

ot variable increase in frequency This variability can in part be explained by the fact hat when the m e.p.p. amplitude is decreased some m.e.p.p. s will be hidden in the noise nd thus the value of the frequency in Triton solution is most likely underestimated.

The time course of individual m.e.p.p. s is seen to be little affected by Triton detergents. As judged from Fig. 2 and Fig. 3, a small increase in the time constant for the decay might e present. This difference was not further examined in the present study

In addition to direct effects of the Triton detergents on the neuromuscular transmission, he resting membrane potential and the input resistance is known to affect the amplitude f the m e.p.p. s (Katz *et al.* 1957 a). The equilibrium potential for the active end-plate is bout 15 mV and the m.e.p p is thus proportional to E 15, *i.e.* the driving force of the n p p. where E is the absolute value of the actual membrane potential. The amplitude f the m.e.p.p.'s is also proportional to the input resistance (R_i) of the muscle fibre. It has arlier been shown that the membrane resistance is little affected by Triton X 100

TABLE I Increase in m p p. frequency after exposure to Triton X 100 (160 μM). Numbers in brackets indicate resting membrane potential.

Fibre	Time of exposure (min)	Frequency Control (s^{-1})	Triton X-100 160 μM (s^{-1})
	12	6 6 (65)	31 6 (−65)
	6	8 6 (92)	10 8 (−92)
	14	0 85 (89)	1.49 (−81)
	5	3 63 (−80)	6.8 (−77)

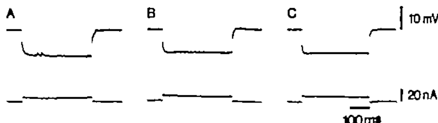


Fig. 4 Effect of Triton X 100 on electrotonic potential changes (upper trace) in response to rectangular current pulses (lower trace) recorded from a muscle end-plate. The current electrode was inserted less than 100 μm from the recording electrode.
A In normal Ringer solution. Input resistance (R_i) = 1.37 MΩ.
B after 15 min in Triton X 100 (160 μM). R_i = 1.20 MΩ.
C: after 30 min in Triton X 100 (160 μM). R_i = 1.1 MΩ.
Resting membrane potential in A −70 mV, B −59 mV and in C −55 mV.

(Rydqvist 1977) but in order to exclude any gross changes in R_i during exposure at high concentrations of detergents R_i was followed in 5 fibres after they had been exposed to 160 10^{-6} M Triton X 100, *i.e.*, the highest concentration used in the present experiments. In Fig. 4 is shown the membrane potential changes due to a 10 nA current pulse in normal Ringer solution (A) and after 15 min (B) and 30 min (C) in 160×10^{-6} M Triton X 100. The electrodes were positioned about 100 μm from each other and the value of R_i is thus only an approximation of the true value, which is calculated by rectangular pulse analysis (*cf.* Boëthius and Rydqvist 1977). The method is, however, useful when comparing effects over a limited time period. It should be noted, however, that R_i cannot be used to estimate changes in the specific membrane resistance (R_m) because of the unknown length constant (λ).

As can be calculated from Fig. 4, R_i = (V/I) in this fibre decreased from a control value of 1.37 MΩ to 1.12 MΩ after 30 min. In 5 cells R_i was decreased by a mean of 14 % (range 0–18 %) which should imply a decrease in m.e.p.p. amplitude of the same amount. In a few cells the R_i was followed after the cell had been returned to normal Ringer solution but only small changes in R_i were seen even when the cell was soaked for up to 60 min. The changes in the resting membrane potential and the R_i are insufficient to explain the decrease in the amplitude of the m.e.p.p.s which must consequently be due to a specific action on the neuromuscular transmission.

Effect on ACh-potentials

The results presented so far suggest that Triton detergents cause a block of the transmission at the end plate. This raises the question whether this is a presynaptic or a postsynaptic effect. It is evident that presynaptic effects must exist from the fact that the frequency of the spontaneous m.e.p.p.s is increased. The decrease in m.e.p.p. amplitude on the other hand can be explained as both pre- and postsynaptic effects. To study the influence of detergent (Triton X 100) on the postsynaptic membrane, iontophoretic application of acetylcholine to the end plate was performed. To ensure that the electrodes were reasonably well in place, several precautions were taken, some of de

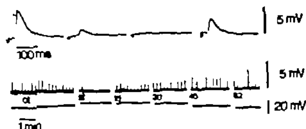


Fig. 5. ACh-potentials from a muscle end-plate showing the effect of Triton X 100 (160 × 10^{-6} M). The iontophoretic pulses (4 ms duration, 5 × 10^{-8} A) were delivered every 14 s and the ACh-potentials recorded (upper and middle traces). Prior to detergent application curare 0.5 × 10^{-6} M was applied (not shown) and the fibre allowed to recover about 43 min. At the first arrow Triton X 100 was added to the Ringer solution and after about 5 min (second arrow) the fibre was returned to normal Ringer solution. The record is later spread at times indicated by numbers below the middle trace. At 60 min the response has increased to about 60% of control amplitude. Inserts above the middle trace show ACh-potentials at different times in an expanded time scale. As is seen the time course of the response is little changed. The lower trace shows the change in resting membrane potential during the experiment. At the end of the experiment the intracellular electrode was withdrawn from the fibre in order to check the E_r (−92 mV). This particular fibre gave small m.e.p.p.'s and was probably a large fibre. The upper and the middle traces are recordings through an AC coupled amplifier (bandwidth 0.1–3 000 Hz).

Methods. Before application of the detergent, the ACh-potentials were recorded during several changes of normal Ringer solution. If stable measurements were obtained, curare (d-tubocurarine) was applied at concentrations varying from about 10^{-6} to 10^{-5} M and the effect and the subsequent recovery was followed. If the ACh-potentials returned to about the precurare amplitude the detergent was applied. In other cases, where the amplitude was considerably reduced or the time course of the ACh-potentials was grossly affected the fibre was discarded. In Fig. 5 is shown responses to iontophoretically applied acetylcholine from an end-plate of the cutaneous pectoris muscle before, during and after application of Triton X-100 (160 × 10^{-6} M). The iontophoretic electrode was placed about 100 µm from the recording electrode and the pulses had a duration of 4 ms and a current of about 5 × 10^{-8} A. The decline in amplitude follows an exponential time course, (τ_{on} = 39 s, see Fig. 6) which is more slow than seen after exposure to curare. Although no quantitative comparison was made the time course seen in the iontophoretic experiments seems to be in accordance with that found in the m.e.p.p. experiments (*cf.* Fig. 1 and 3). After return to normal Ringer solution (second arrow 5 min) the response slowly returned and after 60 min it was about 60% of control value. As shown in the top trace where representative ACh-potentials (at different times) are illustrated in an extended time scale, the time course of the ACh-potentials is approximately the same. In this end-plate the m.e.p.p.'s were small indicating a large fibre. The time needed for recovery is consistent with that as found for m.e.p.p.'s and in the same range found for the recovery of the maximum rate of rise of the action potential in frog muscle. The incomplete recovery can be accounted for if it is assumed that the effect is in part irreversible but it is equally possible that the iontophoretic electrode had been moved or that the fibre was deteriorating. This latter explanation is not probable because the resting membrane potential was found to be −92 mV at withdrawal of the recording micropipette at the end of the experiment.

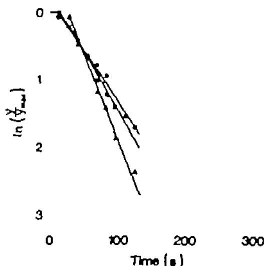


Fig. 6. The time course of the decrease in amplit[illegible] of the ACh-response after application of TX-[illegible] (160×10^{-6} M) for three different end-plates. Absc[illegible] time in sec after application of Triton X 100. Or[illegible] nate ln (y/y_{max}) where y is the amplitude of t[illegible] ACh-response at time t and y_{max} is the amplit[illegible] before Triton X 100 was applied. The times for [illegible] are somewhat arbitrarily chosen because of the [illegible] well defined change of solution (see methods) T[illegible] individual τ_{on} values are 42 s, 59 s and 67 s. τ_{on} estimated from the relation ln $(y/y_{max}) = -(t/\tau_{on})$ This presupposes that the ACh-response is co[illegible] pletely abolished which was not the case for one fib[illegible] (●) (see text).

Another explanation is that the current through the ACh pipette was reduced but that [illegible] probably not the case the resistance of the pipette was only little changed as measur[illegible] before and after the experiment. It is thus obvious that Triton X 100 can alter the respon[illegible] of the membrane potential produced by acetylcholine. This provides evidence th[illegible] Triton X 100 exerts its action on the cholinergic receptor of the postsynaptic membran[illegible]

Kinetic analysis

An estimation of the dissociation constant for Triton X 100 was performed (see Wa[illegible] 1968) using three muscles in which the time courses for the onset of the effect were obtained In Fig. 6 is plotted in a log diagram the reduction in the amplitude of the ACh-potentia[illegible] versus time after 160 10^{-6} M Triton X 100 was applied. In two fibres the time course [illegible] initially exponential, while in the third (●) there is a considerable deviation from [illegible] exponential In this fibre the response was, however not completely abolished but seem[illegible] to reach a steady state value of about 16% of the control value. After subtraction of th[illegible] steady state value the curve was replotted (not shown) an exponential time course was th[illegible] obtained. The mean value from these fibres for the time constant of onset (τ_{on}) is 56 s an[illegible] using the actual concentration 160 10^{-6} M the forward (association) rate constant (k_1) [illegible] 1 1 $\times 10^{4}$ s M This calculation of k presupposes that the time constant for ons[illegible] (τ_{on}) is much shorter than that for offset (τ_{off}) which seems to be justified in the prese[illegible] study (Fulpius 1976) The time constant for offset (τ_{off}) was more difficult to estimat[illegible] because of the virtually irreversible effect of Triton X 100 A rough approximation of 30 mi[illegible] was chosen on basis of several experiments with iontophoretic application (*cf* Fig. [illegible] This gives a value of the dissociation rate constant (k_2) of 5 6 10^{-4} s The dissociatio[illegible] constant ($K_D = (k_2/k_1)$) thus arrived at is 5×10^{-8} M This value should be compared to th[illegible] K_D-values for Triton X 100 of the maximum rate of rise of the muscle action potentia[illegible] which was found to be 35×10^{-6} M using steady state kinetics (Rydqvist 1977). The K_D value for the end plate is probably too low as judged from the very small effect by fo[illegible] example, 40×10^{-6} M and it is probable that the reaction is not of a first order (Ra[illegible]

...ch ...ll be discussed later. In order to estimate the accuracy of the method the dissocia... constant for tubocurarine was calculated using results from three experiments. The ...ues found were k 4.1 10^{4} s^{-1} M (range 2.5–5.2 10^{4} s^{-1} M^{-1}) and k 1.8 10^{-4} s^{-1} ...nge 1.0–2.7 10^{-4} s^{-} M^{-1}) which give a dissociation constant K ~4 10^{-8} M. This is reasonable agreement with values found by other authors (van Maanen 1950, Jenkinson ...50, Highman *et al.* 1963). The method is thus reliable for estimating the kinetic para...ters for Triton but the values should nevertheless be regarded as approximate.

...structural observations

...e electrophysiological findings described above suggest both a presynaptic and a post...synaptic effect produced by the detergent. In order to investigate if morphological changes ...ght accompany these effects a series of muscle preparations soaked for 30 min in ...ton X 100 (160 10^{-6} M) were prepared for electronmicroscopic observation on freeze ...cture replicas as well as in longitudinal ultrathin section.

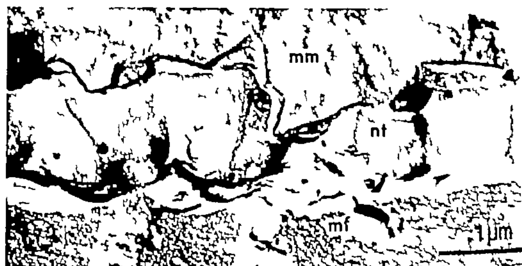
Freeze-fracture replicas. About 10 pairs of cutaneous pectoris muscles were included in ...freeze-fracture study one muscle being soaked in Triton X 100 (160 10^{-6} M) for 30 min, ...twin muscle being used as control. The untreated muscle end-plate had the same ...neral appearance as described by Heuser *et al.* (1974) and by Peper *et al.* (1974). In Fig. 7 ...illustrated a replica of a typical normal end-plate, the nerve terminal (nt) being ...ewed from the outside (PF-side, Branton *et al.* 1976). In the upper part of the picture is ...en the muscle membrane (mm), PF-side, and in the lower part the interior of the ...scle (mf). The terminal is characterized by the elevated ridges traversing the terminal ...ee also Fig. 9) which on both sides are lined with an array of particles arranged as a ...ouble row. The particles have a diameter of approximately 10 nm. In ultrathin sections ...ese ridges are seen as thickenings of the presynaptic membrane described as active zones ...Couteaux *et al.* 1970). The terminal shown in Fig. 7 had also an abundance of smaller ...rticles spread over the terminal membrane. These particles were not always present in ...ormal control end-plates. Such abundance of particles is thought to indicate an active ...d-plate (Heuser 1976) but in the present study where virtually all preparations were ...sting end-plates, this functional correlation was not obvious.

...g. 7 Freeze-fracture view of control nerve terminal from the cutaneous pectoris muscle of the frog fixed ... 2.5 glutaraldehyde and 2 formaldehyde in 0.075 M Na-cacodylate buffer. The nerve terminal (nt) is ...en from the outside (PF-side). On the upper part of the picture is seen the muscle membrane (mm) (PF ...de) and in the lower part seen the interior of the muscle fibre (mf). The presynaptic ridges of the nerve ...terminal are prominent, lined ...th double row of particles (10 nm) on each side. In this terminal particles ... 10 nm) are seen in abundance between ridges. No dimples are seen in the vicinity of the ridges which ...ndicate ...resting nerve terminal. Muscle membrane (mm), nerve terminal (nt), interior of muscle fibre (mf).

...ig. 8 Freeze-fracture view of nerve terminal which have been soaked in Triton X-100 (160 10^{-6} M) for ...30 min and subsequently fixed in 2.5% glutaraldehyde and 2% formaldehyde in 0.075 M Na-cacodylate ...uffer. The view is the same as in Fig. 7. In this terminal several broken away areas of the nerve membrane ...are seen (asterisks). At some distance away from the ridges are seen particles of the same size (arrows) as ...hose lining the ridges. This end-plate was very large and fewer particles are seen between the ridges. Twin ...muscle of that in Fig. 7.

Fig. 9 High magnification of presynaptic ridge from the control terminal seen in Fig. 7.

Fig. 10 High magnification of the Triton exposed terminal membrane seen in Fig. 8. Note clusters of particles away from the ridge (arrows) and broken away membrane patches (asterisks). The particles (arrows) are of the same size as those found lining the ridge (about 10 nm).


mm
nt
mf
1 µm


1 µm


0.5 µm


0.5 µm

replica of the Triton treated twin muscle nerve terminal (Fig. 8 and 10) revealed a more urbed general appearance with several irregular distortions (asterisk) which are probably as where the membrane is broken away displaying the interior of the terminal or the age plane through the membrane might be altered. These distortions were also occa-ally seen in the muscle membrane. The ridges (Fig. 8) are seen somewhat more irregular particles lining the ridges are not arranged in the strict manner seen in normal end-es. In addition clusters of particles (10 nm) are seen at some distance away from the es (arrows). Fig. 10 is an enlarged portion of an area which shows both the extra e particles and the above mentioned distortions. Particle aggregations like these were sionally seen in end-plates soaked in the detergent but never in control muscles.

he postsynaptic muscle membrane, which is characterized by an abundance of intra-mbraneous particles, thought to be the acetylcholine receptor protein and mostly seen he cytoplasmic (PF) side of the membrane was observed in several replicas of both mal and detergent treated muscles, but no obvious difference in the number or angement of these particles could be seen. In summary it thus seems that treatment with ton X 100 induces small or no changes in the postsynaptic membrane, whereas in the synaptic membrane particles of the same size as those lining the ridges appear at some tance away from the ridges, as if lateral diffusion of the particles had occurred.

rathin sections

out 10 pairs of cutaneous pectoris muscles were used, one muscle soaked in Triton 100 (160 10⁻⁴ M) for 30 min, the twin muscle being used as control. After fixation muscle fibres were embedded for longitudinal sectioning. In most cases 1 μm sections, ined with toluidine blue, were first examined in the light microscope to search for end-te regions, and ultrathin sections were subsequently obtained.

In control muscles the neuromuscular junction had the same general appearance as scribed by Birks *et al.* (1960) and by Dreyer *et al.* (1973). However using tannic acid as a rdant usually gave improved preservations of double membranes and in particular the stsynaptic membrane was densely stained (Fig. 11 and 15). In high magnification this embrane was well over 10 nm at the upper portion of the junctional fold, but transformed a considerably thinner membrane seen to outline the base of the fold (Fig. 11 and 15). his is in accordance with earlier findings using horseradish peroxidase-labelled alfa-bunga-

g. 11. Longitudinal section of control end-plate from frog cutaneous pectoris muscle soaked in normal nger solution for 30 min after dissection. The muscle was fixed in 3% glutaraldehyde and 3 para-maldehyde in 0.05 M Na-cacodylate with 2 mM $CaCl_2$ added. Mitochondria (m), synaptic vesicles (sv), ive zone (az), junctional fold (jf).

g. 12. Longitudinal section of an end-plate from the twin cutaneous pectoris muscle of that in Fig. 11 aked in 160 10⁻⁴ M Triton X 100 for 30 min after dissection. Fixation procedure the same as described Fig. 11. Nerve fibre mitochondria (m) are less dense than in the control. Note well defined matrix of uscle mitochondria.

g. 13. Detail of control nerve terminal of Fig. 11 showing mitochondria and synaptic vesicles.

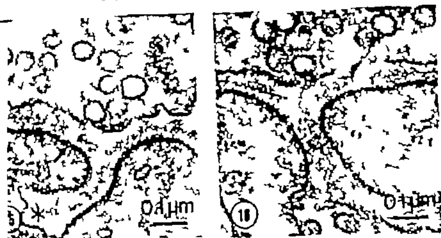
g. 14. Detail of detergent nerve terminal from the same end-plate as in Fig. 12. Triton X-100 (160 10⁻⁴ M) 0 min. The mitochondria are swollen with small internal cristae. Synaptic vesicles are of the same diameter nge as in control (Fig. 13).


m
1 µm


1 µm




1 µm



15 Detail of synaptic junction from the control end-plate in Fig. 11 Note the increased thickening of postsynaptic membrane on the upper part of the junctional fold (arrow) as compared to the thin brane at the base of the junctional fold (asterisk).

16. Equivalent region as in Fig. 15 from Triton exposed end-plate of Fig. 12. The membranes have tially the same appearance as in the control picture (Fig. 15).

ain (Fertuch *et al.* 1974 Daniels *et al.* 1975 Lentz *et al.* 1977). This indicates that ic acid might have a favourable effect on the staining of the postsynaptic membrane ch is rich in acetylcholine receptors. In the nerve terminal, vesicles were clearly outlined the active zones (Couteaux *et al.* 1970) seen as densely stained areas in the presynaptic mbrane over a junctional fold (Fig. 11 and 15). Schwann cell processes are occasionally n intervening the active zones (*cf* freeze-fracture replicas). The mitochondria inside the ve terminal were found to be irregular in shape with somewhat obscured cristae in trast to the mostly well defined cristae in the muscle cells. After the muscle was soaked 160 10^{-4} M Triton X 100, prior to fixation, changes in the general appearance of the d-plate regions were minor However the mitochondria of the nerve terminal were nsiderably more swollen than in control preparations. This was consistently seen in all ffer concentrations used, the general appearance of the mitochondria differing somewhat ig. 11 and 12, Fig. 13 and 14). The mitochondria of the muscle cell were as a rule better served (Fig. 11), at least those found in the vicinity of the end-plate. The reason for this not clear but a diffusion barrier due to the covering of the nerve terminal and its hwann cell might be one cause. A close examination of the pre- and postsynaptic embrane at higher magnification revealed no obvious difference between treated and ntreated muscles (Fig. 15 and 16).

Discussion

The results obtained in the present study show that Triton X-45 as well as Triton X 100 block the neuromuscular transmission but leave the resting membrane potential and membrane resistance relatively unaffected. This block is of postsynaptic origin and is revealed y a decrease in m.e.p.p. amplitude and a decrease in amplitude of iontophoretically

induced ACh-potentials. In addition there is a presynaptic effect, seen as an increase in m.e.p.p. frequency after application of Triton detergents.

Presynaptic effects. In the electron microscope the mitochondria in the presynaptic terminal are noticeably swollen after treatment with Triton X 100 (Fig. 12 and 14). A leakage of Ca^{++} from damaged mitochondria may thus be the cause of the increased m.e.p.p. frequency. The notion that the effect on m.e.p.p. is influenced by mitochondrial membrane activity is supported by the finding that there is a large increase in m.e.p.p. frequency following the addition of dicoumarol, an uncoupler of oxidative phosphorylation (Alnaes *et al.* 1975). Dicoumarol is thought to reduce the active uptake of Ca^{++} which is known to occur (Lehninger 1970) and will lead to an increase in intracellular Ca^{++} concentration. Another compound, ruthenium red, which specifically inhibits Ca^{++} uptake by mitochondria (Moore 1971, Vasington *et al.* 1972) also increases the m.e.p.p. frequency (Rahamimoff *et al.* 1973). Alternative mechanisms should also be considered. For example increased Ca^{++} entry from the extracellular space could give the same effect. It is known that increased Ca^{++} concentration in the external medium increases the m.e.p.p. frequency (Boyd *et al.* 1956, Hubbard 1961, Mambrini *et al.* 1964, Matthews *et al.* 1977).

Another possible action of the detergents is that by their association with the lipid phase of the nerve terminal they increase the fluidity of the membrane, *i.e.*, the freedom of motion of the phospholipid molecules might increase, thereby increasing the probability of fusion between the vesicles and the nerve membrane (Papahadjopoulos *et al.* 1974, 1975 *a, b*, Pagano *et al.* 1975, Van der Bosch *et al.* 1975). Support for this idea is provided by the freeze-fracture observations seen in Fig. 8 and 10, where the nerve membrane is seen with a great number of broken away patches, and particles of the same apparent size as those lining the ridges arranged in clusters at some distant way from the ridges. One explanation for these extraridge clusters might be a lateral diffusion of particles from the ridge due to altered physical properties of the lipid phase.

Postsynaptic effects. By iontophoretic application of ACh during exposure to Triton X 100 it was possible to follow the effect on the postsynaptic membrane. In three cells it was possible to study the recovery and to get an estimate of the time constant for onset and offset of the reaction. The time constant for onset (τ_{on}) of the reaction for the three cells was τ_{on} = 56 s, while the time constant for offset (τ_{off}) of the reaction was estimated to be in the order of 30 min (τ_{off} = 1 800 s) (*cf.* Fig. 5). According to these data the dissociation constant (K_D) for the reaction between Triton X 100 and some postsynaptic site was found to be $5 \times 10^{-}$ M as calculated from the quotient between the dissociation rate constant and the association rate constant (k_1). The K_D value for the effect of Triton X 100 should be regarded with caution because the calculation of this value presupposes that the reaction is of first order, *i.e.*, one molecule of Triton X 100 reacts with one site on the receptor structure. This is probably not true although no quantitative measurements were made of the dose-response relation. Looking at the blocking effect of *e.g.* 40, 80 and 160 $\times 10^{-4}$ M it was obvious that the dose-response curve was rather steep, *i.e.*, the Hill coefficient probably being bigger than one. The effect of 40 $\times 10^{-4}$ M takes very long time to develop and the plausible concentration at which Triton X 100 exert 50% effect on the ACh-potential must be well over 40×10^{-4} M. This does not fit with the K_D value found (5 $\times 10^{-4}$ M).

is assumed that two Triton X-100 molecules simultaneously have to bind to the ptor structure a value for 50% effect of 53 10^{-6} M is obtained which fits better with approximate dose-response relation.
he effect on the postsynaptic membrane is interpreted as an effect on the acetylcholine ptor resulting in an inhibition of the permeability increase, normally following the ction between ACh and the receptor. One possibility is that Triton X 100 has affinity the same sites as ACh, *e.g.* a competitive antagonist, or to other sites on the receptor tein. This cannot be settled without measurements of ACh responses as a function of h output from the iontophoretic pipette, after application of different concentrations of ton X 100. According to the moderate affinity of the detergent to the postsynaptic site action on the stereospecific ACh sites, like curare, seems less probable. An alternative is t the detergent has affinity to other sites which either may mask the ACh sites or modify conformational changes that are assumed to underlie the ionophor activity of the eptor protein and thus inhibit the permeability increase for sodium and potassium Monod *et al.* 1965). It has been shown (Brisson *et al.* 1975) that Triton X 100 decrease response to carbamylcholine at the electroplaque and inhibit the binding of H -nicotine the partially purified receptor protein, in contrast to sodium-dodecyl-sulphate (SDS) other type of detergent which reduces the response to carbamylcholine but does not inhibit nicotine binding. This together with the present findings that the resting membrane ential and the membrane resistance are only little affected give no support for a mechan- n where Triton X 100 interacts with the lipid phase changing it by lyotropic interactions Wojtowicz 1975 Rydqvist 1977) and thereby altering the function of the acetylcholine eptor. However the ultrastructural observations in freeze-fracture replicas and ultrathin ctions show no changes in the postsynaptic membrane which are relevant for the electro- ysiological results even though the resolution, in ultrathin sections, of these membranes is very good using tannic acid as a mordant.

The reversibility of the effect of the detergent is in some doubt. In most cases it was not ssible to obtain the control response after the preparation had been returned to normal nger solution. As mentioned before this could be explained by the considerable time nstant for offset of the reaction, and a general rundown of the fibre which might affect response. Also, the resistance of the iontophoretic micropipette might have been altered ring the recording. It was repeatedly experienced that after a recording from a fibre over period of more than 1 h, impaling another fibre of the same muscle gave perfectly normal sponses including m.e.p.p.s indicating that the action was reversible. Another explana- on is that an irreversible component is present (Brismar and Rydqvist 1978). Close spection of Fig. 6 reveals that more than one time constant seem to be involved in the nset of the reaction between Triton X 100 and the frog end-plate.

In summary both Triton X-45 and Triton X 100 are equally potent in blocking the euromuscular transmission by an effect on the postsynaptic membrane. The results are more easily interpreted in terms of detergent-protein interactions than in terms of detergent lipid interactions, *i.e.* the detergent most likely has a *per se* action on the acetylcholine receptor. The effect is compatible with a drugreceptor interaction with an ED_{50} in the range of 5 to 50 10^{-6} M for Triton X-100, the value being dependent on the type of reaction (Hill

coefficient probably larger than one) The site on the receptor protein to which detergents have affinity has not been possible to determine with the present technique.

The detergents also have a presynaptic effect, the m.e.p.p. frequency being increased probably due to raised Ca^{++} concentration inside the nerve terminal This increase in intracellular Ca^{++} is most likely due to a perturbation of the mitochondrial membrane by the detergent these membranes perhaps being more susceptible to the action of the detergents than the nerve plasma membrane.

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Influence of intravenously administered catecholamines on cerebral oxygen consumption and blood flow in the rat

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Abstract

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order to study effects of catecholamines on cerebral oxygen consumption (CMR_{O_2}) and blood flow (CBF), s undertaken on 75 N_2O and 23 % O_2 were infused i.v. with noradrenaline (2, 5, or 8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) adrenaline (2 or 8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 10 min before CBF and CMR_{O_2} were measured. In about 50 % animals infused ith 2–8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ of noradrenaline, CMR_{O_2} (and CBF) rose. However, there was dose-dependent response, and CMR_{O_2} did not exceed 150 % of control. The effects of noradrenaline in se of 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ on CMR_{O_2} and CBF were blocked by propranolol (2.5 $mg \cdot kg^{-1}$). I animals fused with adrenaline (8 $\mu g \cdot kg^{-1} \cdot min^{-1}$) CMR_{O_2} as doubled and, in many CBF rose 4- to 6-fold. concluded that, hen given in sufficient amounts, catecholamines have pronounced effects on rebral metabolism and blood flow the effects of adrenaline on CMR_{O_2} and CBF resembling those served in status epilepticus.

ey words: CBF CMR_{O_2}, adrenaline, noradrenaline, propranolol

esults reported from this laboratory have shown that, at least in some rat strains, three onditions are associated with an increase in cerebral oxygen consumption (CMR_{O_2}) to bout 200 % of control, and with a marked rise in cerebral blood flow (CBF). These condiions, which are unaccompanied by seizure patterns in the EEG include (1) withdrawal of itrous oxide supply in paralyzed animals in which pain and discomfort were minimized Carlsson *et al.* 1975 1977), (2) amphetamine administration (Berntman *et al.* 1976, 1978), and (3) arterial hypoxia (Berntman *et al.* 1977 Berntman *et al.*, submitted for publication). n the first of these conditions ("immobilization stress"), the increases in CMR_{O_2} and CBF were prevented by adrenalectomy or by prior administration of propranolol, suggesting hat circulating catecholamines were involved (Carlsson *et al.* 1977). This suggestion was supported by results showing that the increase in CMR_{O_2} during hypoxia was considerably reduced by adrenalectomy (Berntman *et al.* 1977).

The objective of the present study was to find out whether systemically administered

catecholamines can increase CMR_{O_2} and CBF to values measured in these three "stress" situations. To that end, paralyzed rats maintained on 70% N_2O were infused i.v. varying doses of noradrenaline and adrenaline. Cortical CBF was measured with technique based on the Fick principle, and CMR_{O_2} was calculated from CBF venous differences in oxygen content.

Methods

Operative and sampling techniques

Male Wistar rats of a S.P.F. strain (Mollegaard Avlslaboratorium, Copenhagen) were anest. 2–3% halothane. When unresponsive the animals were tracheotomized and connected to a Starling respirator delivering gas mixture at a rate that gave arterial CO_2 tensions of 35–40 mmHg. All animals were ventilated with 75% N_2O and 25% O_2. In some 0.6–0.8% halothane was added to the gas mixture during operative procedures but, as soon as these were completed, ventilation was continued on N_2O–O_2 mixture. Body temperature was kept close to 37°C by heating bulb.

Both femoral arteries were cannulated to allow blood pressure recording and sampling of arterial blood for measurements of blood gases, pH, and ^{133}Xenon activity during CBF measurements. One femoral vein was cannulated for infusions of drugs, and the other for infusion of donor blood during rapid sampling of blood for CBF measurements. The caudal portion of the superior sagittal sinus was exposed by a burr hole for sampling of (cortical) cerebrovenous blood.

Infusion of catecholamines

Adrenaline and noradrenaline were obtained as the bitartrates, dissolved in physiological saline at a concentration of 1 mg ml^{-1} (ACO, Stockholm). Before infusion, 0.18, 0.50 or 0.80 ml of the stock solution were mixed with physiological saline to a final volume of 10 ml yielding amine concentrations of 18, 50 and 80 μg ml^{-1} respectively. About 30 min following the completion of operative procedures, adrenaline and noradrenaline solutions were infused at rates of 2, 5 or 8 $\mu g \cdot kg^{-1} \cdot min^{-1}$. The volumes of solution infused were less than 0.5 ml. Some animals were given propranolol (Inderal®) 2.5 mg kg^{-1} i.v. 30 min before CBF measurement. Saturation with ^{133}Xenon was started 10 min before the infusion of catecholamines began. Desaturation was started when the catecholamines had been infused for 10 min. Thus, CBF and CMR_{O_2} values obtained pertain to total infusion period of 10–15 min.

Analytical techniques

Arterial P_{O_2}, P_{CO_2} and pH were measured at 37°C, using microelectrodes of the Eschweiler (Kiel) or Radiometer (Copenhagen) types. All values were corrected for temperature. Arterial and cerebrovenous oxygen content (C_{O_2}) were measured polarographically on 25 μl samples (Fabel and Löbbers 1964, Berntström *et al.* 1974).

Cerebral (cortical) blood flow was measured with ^{133}Xenon modification of Kety and Schmidt (1948) technique, using a partition coefficient for Xenon of 0.83. The basic methodology was as described by Norberg and Siesjö (1974), with the modifications introduced by Berntman *et al.* (submitted for publication). When desaturation was started, and arterial and cerebrovenous samples were collected in rapid succession, donor blood was slowly infused i.v. to maintain blood pressure. Total amount of blood infused was 3–4 ml. Since the CBF (and CMR_{O_2}) obtained is determined mainly by the first 90–120 s of desaturation, the relevant amount infused did not exceed 2 ml. CMR_{O_2} was calculated by multiplying CBF with the arteriovenous difference in oxygen content (AVD_{O_2}). The latter was determined in at least triplicate, the first set of samples being taken just prior to desaturation and the last 2–3 min later. If AVD differed by more than 10% between two consecutive samples, the experiment was discarded. If not, the mean AVD_{O_2} was used to calculate CMR_{O_2}.

Statistical differences were calculated using Student's t test. The following symbols are used: p < 0.05, p < 0.01, p < 0.001

Results

Physiological variables are shown in Table I. Values for body temperature, P_{O_2}, P_{CO_2} and pH were obtained just after CBF measurements. Mean arterial blood pressure was con

...e 1. Body temperature and mean arterial blood pressure (MABP), as well as arterial P_{O_2}, P_{CO_2}, and pH in control rats, and in those infused i.v. with various doses of noradrenaline and adrenaline for 10–13 min.

	Infusion rate $\mu g\ kg^{-1}\ min^{-1}$		Temp. °C	MABP (mmHg)			Pa_{O_2} mmHg	Pa_{CO_2} mmHg	pH
				Preinfus.	Max.	Flow			
trol	—	15	37.0 ±0.1		151 ±4	140 ±3	123 ±5	38.8 ±0.5	7.375 ±0.014
adrenaline	2	6	37.2 ±0.3	148 ±6	178 ±6	143 ±3	105 ±3	37.5 ±1.2	7.299 ±0.024
	5	8	37.8 ±0.4	149 ±5	176 ±8	140 ±5	108 ±3	43.8 ±2.7	7.223 ±0.015
	8	7	37.2 ±0.2	156 ±7	181 ±5	149 ±5	101 ±5	37.7 ±1.3	7.313 ±0.016
renaline	2	5	37.5 ±0.2	146 ±3	167 ±3	141 ±5	012 ±4	38.2 ±1.3	7.307 ±0.030
	8	8	37.0 ±0.2	149 ±6	180* ±6	139 ±5	117 ±5	39.8 ±2.5	7.239 ±0.025

...ues are means ± S.E.M. Control values were taken from Berntman *et al.* (submitted for publication).
... 0.05, $p < 0.01$ p 0.001

...nuously recorded. Values given are those recorded before catecholamines were infused, ...ak pressures obtained during infusion, and values obtained at the beginning of denatura...on.

Some animals infused with *noradrenaline* had moderately elevated temperatures and ...creased CO_2 tensions. Thus, with an infusion rate of 5 $\mu g\ kg^{-1}$ min three animals had ...terial CO_2 tensions of 50, 51 and 53 mmHg, respectively. Peak MABP was close to 180 ...mHg but, during denaturation, values were close to normal. There was a moderate plasma ...idosis. Animals infused with 5 $\mu g\ kg^{-1}$ min after administration of propranolol ...5 mg kg^{-1}) showed similar changes in physiological variables. Arterial P_{O_2} was close ...) or above 100 mmHg in all groups. Arterial oxygen content was similar in all groups ...bout 9 $\mu mol\ ml^{-1}$). Venous oxygen content was decreased in propranolol-treated ...nimals (from a control value of 6.07 ± 0.23 to 4.69 ± 0.43 $\mu mol\ ml^{-1}$).

Animals infused with *adrenaline* in dose of 2 $\mu g\ kg^{-}$ min showed values similar to ...hose found in animals infused with noradrenaline. With an infusion rate of 8 $\mu g\ kg^{-}$ min^{-1} ...MABP was elevated at the time of denaturation. Two animals had increased CO tensions ...(50 and 52 mmHg, respectively). Mean venous oxygen content was significantly increased ...(from a control value of 6.07 ± 0.23 to 7.28 ± 0.44 $\mu mol\ ml^{-1}$).

Fig. 1 shows individual CBF and CMR_{O_2} values obtained during infusion of noradrena...line. With 2 $\mu g\ kg^{-1}\ min^{-1}$ three CMR_{O_2} values of six were increased above control and, after 5 $\mu g\ kg^{-1}\ min^{-1}$ CMR_{O_2} rose in at least five of eight animals. Results obtained with ...an infusion rate of 8 $\mu g\ kg^{-1}\ min^{-1}$ indicate that CMR_{O_2} did not increase in proportion to dose ("all or none response"). There was considerably scatter in CBF values. Marked increases were observed only in the three hypercapnic animals and, in many the increase in CBF was small or moderate.

Fig. 2 shows that, in propranolol-treated animals, infusion of noradrenaline (5 $\mu g\ kg^{-1}$

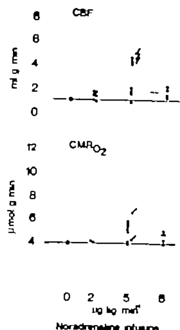


Fig. 1

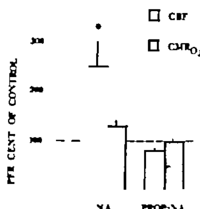


Fig. 2

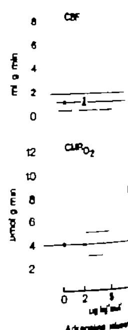


Fig. 3

Fig. 1 Individual CMR$_{O_2}$ and CBF values measured in animals infused with various doses of noradrenaline. Mean control values are given by large filled circles, and ±2 S.D by dotted lines. Values obtained in hypercapnic animals (Pa_{CO_2} 50–53 mmHg) are denoted by arrows. Note absence of dose-dependent response

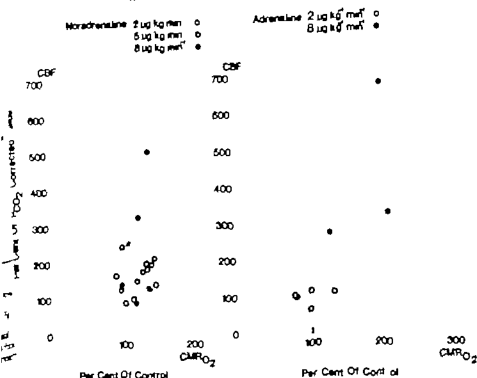
Fig. 2. Influence of propranolol (2.5 mg kg⁻ given i.v 30 min prior to CBF measurements) on changes in CBF and CMR$_{O_2}$ (in percent of control) following infusion of noradrenaline (5 µg·kg⁻ min⁻¹). Values obtained without propranolol during noradrenaline (NA) infusion are given to the left, those obtained in propranolol-treated animals to the right. (Prop+NA) $p<0.01$

Fig. 3 Individual CBF and CMR$_{O_2}$ values measured in animals infused with 2 or 8 µg kg⁻¹ min⁻¹ adrenaline. Mean control values are given by large filled circles, ±2 S.D by dotted lines. Values obtained in hypercapnic animals (Pa_{CO_2} 50–52 mmHg) are denoted by arrows.

min ¹) was unaccompanied by increases in CMR$_{O_2}$ and CBF In fact, there was a suggestive decrease in CBF

Infusion of adrenaline had effects different from those observed after noradrenaline (Fig. 3) With 2 µg kg⁻ min only one CMR$_{O_2}$ value was increased above control, while 8 µg kg⁻ min gave rise to an excessive rise in oxygen consumption. Thus, in six of eight animals CMR$_{O_2}$ exceeded 8.2 µmol g⁻ min (200% of control) and only two had CMR$_{O_2}$ values in the range observed with infusion of noradrenaline In these two animals CBF was only moderately increased (1 7 and 2.5 ml g⁻ min respectively) but in the majority of the remaining animals, CBF increased 3- to 6-fold In the latter group, two animals had CBF values of 5 7 and 7.3 ml g⁻ min in spite of the fact that arterial P_{CO_2} was lower than 40 mmHg.

In order to allow a comparison of increases in CBF and CMR$_{O_2}$ during infusion of noradrenaline and adrenaline all individual CBF values were expressed as per cent of the value, expected at their measured P_{CO_2}, assuming a 5% change in CBF per mmHg change in P_{CO_2} (Norberg and Siesjö 1974) The results are shown in Fig. 4 Following noradrenaline infusion, there was a relatively poor correlation between increases in CMR$_{O_2}$



[illegible] 4 Relationship between increases in CMR_{O_2} and CBF in animals infused with noradrenaline and [illegible]enaline. The values are expressed in percent of control values. In deriving percentage changes from [con]trol, CBF values were first corrected. Each individual CBF value is related to the control value, expected [at] the actual P_{CO_2}, assuming [illegible] 5 [illegible] change in CBF per mmHg change in P_{CO_2}.

[a]nd CBF but, in the majority of animals, there was a proportionally larger rise in CBF [t]han in CMR_{O_2}. This tendency was also observed in adrenaline-infused animals and, in two [a]nimals, CBF increased 6-fold for a 2-fold rise in CMR_{O_2}.

Discussion

In interpreting the present results, one difficulty arises from the fact that catecholamines are known to poorly penetrate the blood-brain-barrier (Weil-Malherbe *et al.* 1961 Bertler *et al.* 1966, Oldendorff 1971). Data published in the literature on cerebral metabolic and circulatory effects of systemically administered catecholamines are inconsistent. The first clear indication that catecholamines may affect cerebral metabolism and blood flow in man was reported by King *et al.* (1952) who found that large doses of adrenaline *i.v.* induced a 20% rise in CMR_{O_2} and CBF whereas noradrenaline in similar doses only caused a small reduction in flow A subsequent study of equally large doses of adrenaline (given *i.m.*) failed to corroborat the effects on CMR_{O_2} and CBF (Sensenbach *et al* 1953). However like King *et al* (1952) these authors noted that noradrenaline gave rise to a reduction in CBF and several other groups have obtained comparable results (*e.g* Greenfield and

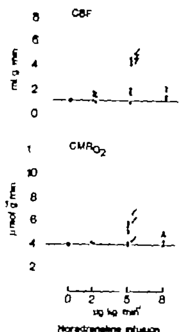


Fig. 1

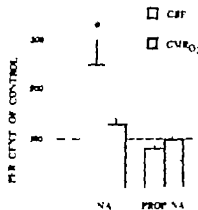


Fig. 2

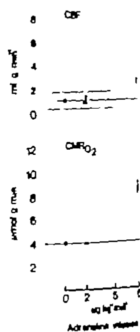


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In order to allow a comparison of increases in CBF and CMR_{O_2} during infusion of noradrenaline and adrenaline all individual CBF values were expressed as per cent of the value, expected at their measured P_{CO_2}, assuming a 5% change in CBF per mmHg change in P_{CO_2} (Norberg and Siesjö 1974). The results are shown in Fig 4 Following noradrenaline infusion, there was a relatively poor correlation between increases in CMR

results obtained on CBF following administration of noradrenaline and adrenaline are compatible with the presence of a constrictory α-adrenergic effect (see Ekström-Jodal et al. 1974, McDonnel et al. 1975), which is variably superimposed on the vasodilatory effect, the latter possibly being coupled to an adrenergic effect on metabolism. If it is assumed that adrenaline has equally strong effects on α- and β-receptors while noradrenaline [is] dominating α-effects (and a weak β influence), it is possible to explain both the metabolic and the circulatory effects observed presently. It is of interest that in three animals [inf]used with noradrenaline, moderate hypercapnia (P_{CO_2}, 50–53 mmHg) was associated with marked rise in CBF. Possibly these results reflect the fact that hypercapnia blocks [α]-receptor activity without influencing β-receptor stimulation (see, however, McDonnel et al. 1975).

The present results leave open the question how circulating catecholamines exert their [eff]ects on cerebral metabolism and blood flow. In view of present knowledge on blood-[br]ain barrier permeability to catecholamines (see Introduction) a direct cerebral effect could [eit]her be due to penetration into areas with an imperfect barrier or to an "opening" of the [in]tact barrier [...] due to the increase in blood pressure. Results supporting the latter [alt]ernative have been published (MacKenzie et al. 1976, Hardebo et al. 1977). We are [pr]esently studying whether increases in CMR_{O_2} and CBF correlate with signs of blood-[br]ain barrier dysfunction. However, whatever is the mechanism, the present results show that [ca]techolamines have pronounced effects on CMR_{O_2} and CBF and it is of considerable [in]terest that adrenaline may increase CMR_{O_2} to values close to those observed [d]uring status epilepticus (see Meldrum and Nilsson 1976, Blennow et al. 1978).

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Tindall 1968, Ekström-Jodal *et al* 1974) In contrast, Olesen (1972) concluded that neith noradrenaline nor adrenaline, when given by intracarotid infusion in unanesthetized m had any significant effect on CBF

Recent experimental results demonstrate that, at least under some circumstances, system cally administered catecholamines may significantly influence CMR_{O_2} and CBF Res obtained in dogs by Xanalatos and James (1972) and by McDonnel *et al* (1975) led to th conclusion that *i v* infusion of isoprenaline and noradrenaline increase CMR_{O_2} and CBF a β-receptor mediated effect. This effect which was assumed to be elicited from chemoreceptors, seemed to oppose a direct vasoconstrictory action (of noradrenaline) α receptor stimulation. The method used by the authors may not have been well suited studies of CMR_{O_2} and since barbiturate anesthesia was used, they leave open the quest whether catecholamines can increase CBF and CMR_{O_2} above unanesthetized cont values. Recent data on monkeys (MacKenzie *et al* 1976) clearly demonstrate that n adrenaline may increase CMR_{O_2} and CBF However such effects were obtained o when noradrenaline was infused intracarotidally following prior "osmotic opening of t blood-brain barrier or if the amine was injected intraventricularly The authors conclud that noradrenaline augments cerebral metabolic rate if it passes the blood brain barr and that the increase in CBF was secondary to activation of metabolism.

As stated, the objective of the present experiments was to study whether intravenou administered catecholamines can increase CMR_{O_2} and CBF to values previously obser in situations that must be considered stressful In interpreting the results it should b recalled that the anaesthesia used (70% N_2O) does not by itself reduce CMR_{O_2} bel normal (Carlsson *et al* 1976) and that the CBF method used can be considered quantitat as it is based on the Fick principle. The results amply confirm those of previous investigat (McDonnel *et al* 1975 MacKenzie *et al* 1976) in showing that noradrenaline increas CMR_{O_2} and CBF and the percentage increases observed presently are not different fr those reported by these authors. However our results demonstrate that, during noradre line infusion there was a considerable variability in response, and CMR_{O_2} did not rise abo 150% of control. In contrast, when given in sufficient amounts, adrenaline provoked doubling of CMR_{O_2} with some CBF values reaching 500–600% of control. The increase CMR_{O_2} following adrenaline infusion is similar to that previously observed in "immobili tion stress" in hypoxia, and following amphetamine administration (see Introduction), a the peak CBF values resemble those seen in animals given amphetamine. It is thus tempt to conclude that at least in the first two of these three situations, the changes observed cerebral metabolic rate and blood flow were elicited by circulating adrenaline. Su excessive changes have not been observed in all animal strains studied (see Berntman *et* 1977 1978). By analogy we conclude that failure of observing a marked increase in CM (or CBF) either was due to variations in release of adrenaline, or in its passage from blo to tissue. It should be recalled that this variability in response also has been observed in m (King *et al* 1952, Sensenbach *et al* 1975)

Our results confirm those of others (Xanalatos and James 1972, McDonnel *et al* 19 MacKenzie *et al.* 1976) in showing that the influence of catecholamines on CMR_{O_2} (a CBF) is blocked by propranolol, suggesting that β-receptor stimulation is responsib

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Metabolic adaptation to hypoxia

Redox state of the cellular free NAD pools, phosphorylation state of the adenylate system and the (Na^+–K^+)-stimulated ATP-ase in rat liver

By

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Abstract

KINNULA, V. L. and I. HASSINEN. *Metabolic adaptation to hypoxia. Redox state of the cellular free NAD pools, phosphorylation state of the adenylate system and the (Na^+ K^+)-stimulated ATP-ase in rat liver.* Acta physiol. scand. 1978. *104* 109–116.

The effect of hypoxia (30 min 10 % or 8 % O_2) on the phosphorylation state and redox state of the cytosol and mitochondria of rat liver were studied. Measurements were made both from normal animals and rats which had been exposed to the reduced partial pressure of oxygen (50.5 kPa or 40.8 kPa of air) for one or seven days. Cytosolic free NAD was reduced in the liver both in acute hypoxia and in hypoxia after one or seven days, i.e. the lactate/pyruvate and α-glycerol-3-phosphate/dioxyacetonephosphate ratios increased markedly. A marked reduction in the mitochondrial free NAD pool occurred only in acute hypoxia and only a slight reduction was observed in animals kept at 40.8 kPa for one or seven days, as evaluated from the hepatic hydroxybutyrate/acetoacetate ratio. Liver ATP concentration decreased rapidly in acute hypoxia without any significant recovery during one or seven days at 40.8 kPa. The hepatic ATP/ADP · P ratio decreased significantly with a simultaneous decrease in the total adenine nucleotide concentration. A tendency was observed for the ATP/ADP · P ratio to return to normal after seven days. The values in acute hypoxia were significantly smaller than those noted in hypoxia after seven days, demonstrating an adaptation of the energy metabolism during prolonged hypoxia. Hepatic (Na^+ K^+)-stimulated ATP-ase activity was not affected by hypoxia.

Key words: *Liver hypoxia, redox state of free NAD pool, phosphorylation state of the adenylate system,* (Na^+–K^+)-ATP-ase, adaptation to hypoxia

By measuring the ratio of the concentrations of oxidized and reduced substrates of a suitable, compartmentalized NAD-linked dehydrogenase, the redox state of the cytosol and mitochondria can be calculated separately (Bücher and Klingenberg 1958). Acute ischemia of the liver achieved by clamping the hepatic artery and portal vein for 60 sec (Chance *et al.* 1965), and hypoxia of the animal (7 % O_2 in the inhaled air) for 30 min (Lal and Miller 1973) result in an increase in the cytosolic NADH/NAD ratio and decrease in the ATP/ADP ratio of the liver (Chance *et al.* 1965). Anoxia results in a decrease in the hepatic ATP/ADP ratio, but the magnitude of the decrease and survival time in anoxia is dependent on the type of ... (Rähä 1964, Mäenpää 1967)

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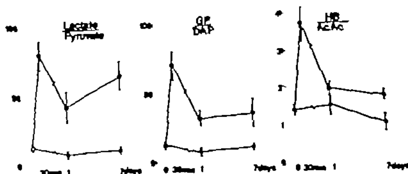


Fig. 1. Lactate/pyruvate, α-glycerol-3-phosphate/dihydroxyacetone phosphate and 3-hydroxybutyrate/acetoacetate ratios in the liver after various periods in moderate hypoxia. The animals were in the respirator 30 min under 10% O_2. In the 1-day and 7-day experiments they were exposed to high altitude effects (50.5 kPa) for the given length of time before the 30 min period in the respirator under 10% O_2. The results are means ± S.E. from 4–10 separate experiments. Symbols: ○, control; ●—● hypoxic and ■—■ pair ±; GP α-glycerol-3-phosphate; DAP dihydroxyacetone phosphate; HB, 3-hydroxybutyrate and AcAc, acetoacetate.

...ve irreproducible results, membrane fractions were isolated as follows: The homogenate was centrifuged at 1 000 *g* for 10 min and the supernatant further centrifuged at 6 000 *g* for 15 min. This supernatant was then centrifuged at 48 000 *g* for 30 min. The pellets from the 1 000 *g* and 48 000 *g* centrifugations were homogenized with the sucrose medium and diluted fourfold with water. The reaction was started by the addition of 0.2 ml of the homogenate, allowed to proceed for 4 min at 37°C, and stopped with trichloroacetic acid, and the inorganic phosphate (P_i) was determined as described by Ernster *et al.* (1950). The (Na^+–K^+)-stimulated ATP-ase activity was taken as the difference between the total ATP-ase activity and that observed in the presence of ouabain and the absence of NaCl and KCl. The values for the 1 000 *g* sediment were not reproducible, and therefore only the 48 000 *g* activities are reported here. The results are expressed as µmol of P_i/min per g wet weight of liver and nmol of P_i/min per mg protein. Protein was determined by the method of Lowry *et al.* (1951).

To test the validity of the method used, a single intraperitoneal dose of L-thyroxine (6 mg/kg in 10 mM NaOH–0.9% NaCl) was administered to rats, which were then killed 24 hours later. Whilst the (Na^+–K^+)-stimulated ATP-ase activity in the 48 000 *g* sediment of the liver homogenate was 21.1 ± 7.5 (mean ± S.D., n = 3) nmol P_i/min/mg protein, that in the thyroxine-treated animals was 36.2 ± 15.1 (mean ± S.D., n = 2). Thus the method is sensitive enough to detect hormone-dependent changes.

Serum thyroxine determinations. Serum thyroxine (T_4) was determined by the modified method of Chopra (1972). A 20 µl sample of serum was incubated overnight at 4°C with 100 µl of antiserum in boric acid, BSA, 8-anilino-1-naphthalenesulphonic acid solution, pH 8.5, and 100 µl (10 000 cpm) of [^{125}I]-thyroxine solution (in boric acid, BSA solution, pH 8.5), the final medium containing 0.2 M boric acid pH 8.5, BSA g/l and 8-anilino-1-naphthalenesulphonic acid 1.9 g/l. The immunocomplex was precipitated with polyethyleneglycol (in 0.9% NaCl) to the final concentration of 12.5% (w/v) (Henquin et al. 1974).

Statistical treatment. The two-tailed student's t-test for independent means was used for the statistical analysis, and P values of < 0.05 were considered to indicate statistical significance.

Results

Lactate/pyruvate ratio. This ratio showed a significant increase in acute hypoxia (Fig. 1). After 30 min under 10% O_2 in the respirator the ratio increased by a factor of 6.0 ($p < 0.001$), and the ratio was elevated significantly by a factor of 3.2 ($p < 0.05$) after one day and 4.8 ($p < 0.001$) after seven days at 50.5 kPa following 30 min in the respirator under approximately the same partial pressure of oxygen (Fig. 1). The change in the lactate/pyruvate

Thyroxine administration increases the mitochondrial cytochromes, and certain enzymes of the oxidative metabolism (Kadenbach 1966). The hypermetabolic states of the l resulting from thyroxine treatment, chronic ethanol administration or cold acclimation connected with increased (Na^+-K^+)-stimulated ATP-ase activity and the extra O_2 consumption noted under these conditions seems to be due to the increased ATP consumption this enzyme (Ismael Beigi and Edelman 1971 Israel *et al* 1973 Bernstein *et al* 1975, also Israel *et al* 1975). Hypoxia is a condition reported to result in decreased thyroid function (Galton 1972) and therefore changes in the (Na –K^+)-stimulated ATP-ase may be significance in regulating ATP-turnover in low oxygen tension

Material and methods

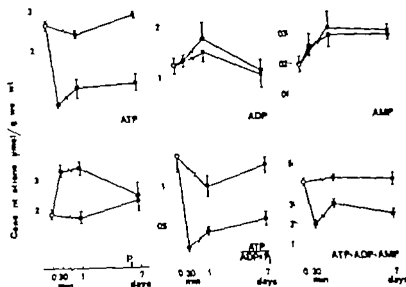
Reagents. Standard reagents were obtained from E. Merck AG Darmstadt, Germany Lactate dehydrogenase, glycerol-3-phosphate dehydrogenase, hydroxybutyrate dehydrogenase and the co-enzymes purchased from Boehringer GmbH, Mannheim, Germany and pyruvate kinase, glucose-6-phosphate dehydrogenase hexokinase and myokinase from Sigma Chemicals, St. Louis, Mo U.S.A. L-thyroxine and bovine serum albumin (BSA) were purchased f om Sigma Chemical Co., St. Louis, Mo US L [3 ,5 ^{125}I]thyroxine specific radioactivity 20–50 mCi/mg, was obtained from the Radiochemical Ce Amersham, Bucks, U K., thyroxine antiserum from Farmos Ltd., Finland, 8-amino-1-naphtalene-sulphonic acid from Kodak Eastman Chemicals, Rochester N Y U.S.A. and polyethyleneglycol from Fluka Buchs, Switzerland.

Animals and hypoxic experiments Sprague Dawley rats from the Department's own colony were Both sexes were used and the ages of the animals varied between 3 to 4 months. Their food consisted standard rodent chow (Astra-Ewos AB, Södertälje, Sweden). The animals were in each case divided hypoxic, pair-fed control and *ad libitum* control groups.

F r the metabolite determinations the rats were anesthetized with pentobarbital (60 mg/kg, intraperitoneally), the trachea was cannulated and respiration controlled with a rodent respiration pump (Harvard Apparatus Company Model 680). The respiratory rate was fixed at 60 cycles per min, and the tidal volume calculated from the equation log = – 1.57 + 0.735 log m, where m is the body weight of the animal grammes and v the tidal volume in millilitres (Kleinman and Radford 1971). The control animals breathed room air for 30 min nd the hypoxic animals 10 % O_2 or 8 O_2 in nitrogen. In one group of hypoxic experiments the animals were transferred to the respirator (10 % O_2) for 30 min from a barometric pressure chamber in which they had been exposed to 50.5 kPa for one to seven days, these are referred to as the moderate hypoxia experiments. In another group of experiments the animals were taken to the respirator (8 % O_2) from the barometric pressure chamber in which they had again been from one to seven days, time (40.8 kPa, these being referred to as severe hypoxia experiments. I studying (Na K)-stimulated ATP-ase ctivity in hypoxia, only the hypobaric pressure chamber wa used.

Metabolite determinations. At the end of the 30 min controlled ventilation in the respirator one lobe the liver was freeze-clamped according to Wollenberger *et al.* (1960). The samples for the metabolite determinations were pulverized in a mortar under liquid nitrogen. Approximately 0.5 g of frozen tissue powder was extracted twice with 3 ml of ice-cold 6 perchloric acid. After the centrifugations the combined supernatant was neutralized (pH 6.0 with 3 75 M K_2CO_3 containing 0.5 M triethanolaminehydrochloride Neutralized perchloric acid extracts were used for enzymatic assays of lactate (Gawehn and Bergmeyer 1970), pyruvate (Czok and Lamprecht 1970), sn-glycerol-3-phosphate (Hohorst 1970), dihydroxyacetone phosphate (Bücher and Hohorst 1970), 3-hydroxybutyrate (Williamson nd Mellanby 1970), acetoacetate (Mellanby and Williamson 1970), adenosine 5-triphosphate (Lamprecht and Trautschold 1970), adenosine 5-diphosphate (Jaworek *et al.* 1970) denosine-5-monophosphate (Jaworek *et al* 1970) nd inorganic phosphate (Chen 1956).

(Na^+ K)-stimulated ATP-ase For the determination of (Na K)-stimulated ATP-ase (EC 3 6 1 the rats were decapitated after 1 or 7 days at 40.8 kPa and their livers rapidly transferred to ice-cold 0.32 sucrose containing 1 mM EDTA Tris buffer pH 7 4. The determination of ATP ase proceeded essentially according to Bernstein *et al.* (1973). A 600 mg liver sample was homogenized with the same medium i glass homogenizer with a motor-driven Teflon pestle. Because activity determinations of total homogenates



3. Adenine nucleotide concentrations and phosphorylation state in the liver after various periods in ... hypoxia (40.8 kPa and ... O_2). Experimental conditions and symbols as in Fig. 1. Values are means ± ... from 4–6 separate experiments. ATP adenosine-5-triphosphate; ADP adenosine-5-diphosphate; ...P adenosine-5-monophosphate; P inorganic phosphate; ATP/ADP P phosphorylation state.

effect on the ratio though both the hydroxybutyrate and acetoacetate concentrations ...re increased especially after complete fasting for 4 h (data not shown).

Adenine nucleotides. The results are presented in Fig. 3. In acute severe hypoxia the ATP ...ncentration decreased ($p < 0.001$), and this decrease was still significant in chronically ...poxic animals after seven days ($p < 0.01$). No significant differences were observed be...een these acutely and chronically hypoxic animals ($p < 0.1$). A simultaneous increase ... 0.01) in the inorganic phosphate concentration was observed in hypoxia. The ADP ...d AMP concentrations did not change significantly in any instance. The phosphoryla...on state (ATP/ADP P) decreased both in acute ($p < 0.001$) and chronic hypoxia of ...ven days ($p < 0.02$), though there was also a significant difference between these two values ... 0.01). The total amount of hepatic adenine nucleotides decreased ($p < 0.001$) in acute ...ypoxia, no differences being observable between these values and those recorded after ...ypoxic periods of one or seven days.

(Na^+–K^+)-stimulated ATP-ase activity in the 48 000 g sediment did not change significantly ...n hypoxia after one day at 40.8 kPa. It was lower after 7 days in hypoxia when compared ...with the *ad libitum* control values, but the difference between the hypoxic and the pair-fed ...control activities was not significant (Table I).

The serum T concentration was 45.8 ± 4.0 nmol/l in the *ad libitum* control group, 49.3 ± 9.4 ...n the pair-fed control group and 35.6 ± 4.4 in the hypoxic group after seven days at 40.8 ...kPa. These values are means ± S.D. from 7–8 different experiments, and the significance ...between the *ad libitum* control and hypoxic values is $p < 0.1$ and between the pair-fed and ...hypoxic values p 0.05

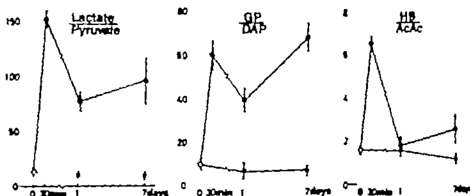
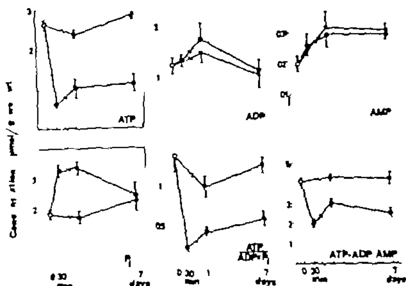


Fig. . Lactate/pyruvate, *sn*-glycerol-3-phosphate/dioxyacetone phosphate and 3-hydroxybutyrate acetate ratios in the liver after various periods in severe hypoxia (40.8 kPa and 8% O_2). Experimental conditions and symbols as in Fig. 1. Values are means ± S.E. from 4–5 separate experiments.

ratio was mainly due to change in the lactate concentration (see Fig. 1). The hepatic pyru concentration was 0 137 ±0 003 (mean ± S D., n = 9) μmol/g wet weight in the controls 0 098 ±0.041 (mean ± S.D n = 6) after 30 min of hypoxia (10% O_2). In severe hypoxia lactate/pyruvate ratio increased by a factor of 11.2 ($p < 0.001$) after 30 min under 8 and 5 8 ($p < 0.001$) after one day and 7.0 ($p < 0 001$) after seven days at 40.8 kPa prior to 30 min period in the respirator under 8% O_2 (Fig. 2). The hypoxic ratios were smaller one day or seven days at 50.5 kPa or 40 8 kPa than in acute hypoxia with a correspon partial pressure of oxygen When the animals were removed from 40 8 kPa to 10% instead of 8% O_2, the results were about the same as at 50.5 kPa followed by 30 min und 10% O_2. Pair-feeding, in effect food restriction of the animals, resulted in no signific changes in the ratios (Fig. 1 and 2)

sn-Glycerol 3-phosphate/dioxyacetonephosphate ratio The ratio increased by a factor 5.6 ($p < 0.001$) in acute moderate hypoxia and by 2.8 ($p < 0.05$) after seven days in moder hypoxia, this figure not being significantly different from the acute value ($p < 0.1$). The ra was lower after the adaptation period of 24 h than in acute hypoxia, however (p < 0 (Fig. 1). The dioxyacetonephosphate concentration remained almost constant in spite large changes in the *sn*-glycerol-3-phosphate/dioxyacetonephosphate ratio. The hepa dioxyacetonephosphate concentration was 0 043 ±0 007 (mean ± S D n = 10) μmol/g w weight in the control animals and 0 038 ±0.010 (mean ± S D., n = 8) in the animals kept i hypoxia (10% O_2) for 30 min. The transient change in the ratio noted during the first d in severe hypoxia did not prove significant ($p < 0.1$) (Fig. 2).

3-hydroxybutyrate acetoacetate ratio The ratio increased by a factor of 2.7 ($p < 0.05$) acute hypoxia under 10% O_2, and 4 2 ($p < 0 001$) under 8% O_2. In acute hypoxia the increa in the 3-hydroxybutyrate/acetoacetate ratio was due to an increase in the 3-hydroxybutyra concentration. The hepatic acetoacetate concentration was 0.035 ±0 004 (mean ± S.I n = 4) μmol/g wet weight after 30 min in hypoxia (10% O) and 0 039 ±0.005 (mean ± S.I n = 4) in the control animals. During chronic hypoxia the ratio decreased permanently near the control values, and no differences between the control and hypoxic ratios could observed after either one day or seven days of adaptation (Fig. 1 and). Pair-feeding h



3. Adenine nucleotide concentrations and phosphorylation state in the liver after various periods in hypoxia (40.8 kPa and 8 ... O_2). Experimental conditions and symbols as in Fig. 1. Values are means ± ... from 4–6 separate experiments. ATP adenosine-5-triphosphate; ADP adenosine-5-diphosphate; AMP adenosine-5-monophosphate; P_i inorganic phosphate; ATP/ADP·P_i phosphorylation state.

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Adenine nucleotides. The results are presented in Fig. 3. In acute severe hypoxia the ATP concentration decreased (p 0.001), and this decrease was still significant in chronically hypoxic animals after seven days ($p < 0.01$). No significant differences were observed between these acutely and chronically hypoxic animals ($p < 0.1$). A simultaneous increase (p 0.01) in the inorganic phosphate concentration was observed in hypoxia. The ADP and AMP concentrations did not change significantly in any instance. The phosphorylation state (ATP/ADP·P_i) decreased both in acute ($p < 0.001$) and chronic hypoxia of seven days ($p < 0.02$), though there was also a significant difference between these two values (p 0.01). The total amount of hepatic adenine nucleotides decreased ($p < 0.001$) in acute hypoxia, no differences being observable between these values and those recorded after hypoxic periods of one or seven days.

(Na^+–K^+)-stimulated ATP-ase activity in the 48 000 g sediment did not change significantly in hypoxia after one day at 40.8 kPa. It was lower after 7 days in hypoxia when compared with the *ad libitum* control values, but the difference between the hypoxic and the pair-fed control activities was not significant (Table I).

The serum T_3 concentration was 45.8 ± 4.0 nmol/l in the *ad libitum* control group 49.3 ± 9.4 ... the pair-fed control group and 35.6 ± 4.4 in the hypoxic group after seven days at 40.8 kPa. These values are means ± S.D. from 7–8 different experiments, and the significance between the *ad libitum* control and hypoxic values is $p < 0.1$ and between the pair-fed and hypoxic values p 0.05.

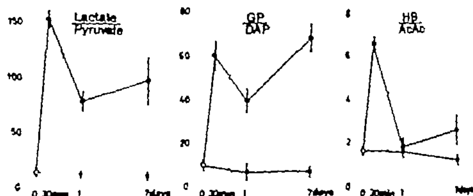


Fig. 2. Lactate/pyruvate sn-glycerol-3-phosphate/dio yacetone phosphate and 3-hydroxybutyrate/aceto acetate ratios in the liver after various periods in severe hypoxia (40.8 kPa and 8 % O_2). Experimental conditions and symbols as in Fig. 1 Values are means ± S.E. from 4-5 separate experiments.

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sn-Glycerol-3-phosphate/dioxyacetonephosphate ratio The ratio increased by a factor of 5 6 ($p < 0.001$) in acute moderate hypoxia, and by 2.8 ($p < 0.05$) after seven days in moderate hypoxia, this figure not being significantly different from the acute value ($p < 0.1$). The ratio was lower after the adaptation period of 24 h than in acute hypoxia, however ($p < 0.05$) (Fig. 1). The dioxyacetonephosphate concentration remained almost constant in spite of large changes in the sn-glycerol-3-phosphate/dioxyacetonephosphate ratio The hepatic dioxyacetonephosphate concentration was 0.043 ± 0.007 (mean ± S D n = 10) µmol/g wet weight in the control animals and 0 038 ± 0.010 (mean ± S.D., n = 8) in the animals kept in hypoxia (10 % O_2) for 30 min. The transient change in the ratio noted during the first day in severe hypoxia did not prove significant ($p < 0.1$) (Fig. 2).

3-hydroxybutyrate acetoacetate ratio The ratio increased by a factor of 2.7 ($p < 0.05$) in acute hypoxia under 10 % O_2, and 4.2 ($p < 0.001$) under 8 % O_2. In acute hypoxia the increase in the 3-hydroxybutyrate/acetoacetate ratio was due to an increase in the 3-hydroxybutyrate concentration. The hepatic acetoacetate concentration was 0 035 ± 0.004 (mean ± S.D., n = 4) µmol/g wet weight after 30 min in hypoxia (10 % O_2) and 0 039 ± 0 005 (mean ± S.D., n = 4) in the control animals. During chronic hypoxia the ratio decreased permanently to near the control values, and no differences between the control and hypoxic ratios could be observed after either one day or seven days of adaptation (Fig. 1 and 2). Pair-feeding

was observed during seven days in hypoxia by measuring the serum T_4 concentration. This observation is in agreement with the increased phosphorylation state during the adaptation period, since a decreased phosphorylation state has been previously observed in the hepatic adenylate system of thyroxine-treated rats by Hassinen *et al.* (1971).

The most sensitive indicators for the effects of hypoxia appear to be the decrease in ATP content and ATP/P ratio. Variations in ADP and AMP content cannot be interpreted with certainty because of the rapid decomposition of these nucleotides. The total adenine nucleotide concentration (ATP + ADP + AMP) decreased in hypoxia. The degradation of adenine nucleotides beyond AMP is dependent on the activity of 5-nucleotidase and/or AMP-deaminase, leading to adenosine or IMP and in both cases eventually to inosine. Since ATP inhibits 5-nucleotidase (Baer *et al.* 1966) and inorganic phosphate inhibits AMP-deaminase (Nikiforuk and Colowick 1956, Ronca-Testoni *et al.* 1970) the route via adenosine would be favoured in hypoxia. Adenosine, one of the most potent vasodilators, is active in the ischemic myocardium (Rubio *et al.* 1973). The degradation products of IMP and adenosine lead to inosine, hypoxanthine, xantine and allantoin, and it is by this mechanism that the increased concentration of inosine in anoxia can be explained (Olsson 1970).

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TABLE I (Na⁺ K⁺)-stimulated ATP-ase activity in control and hypoxic liver fractions (40 000 g sed The hypoxic periods consisted of one or seven days at 40.8 kPa. Values are means ± S D the number of experiments indicated in parentheses.

	Activity	
	nmol/min/g wet wt.	nmol/min/mg prot.
Control		
fed ad lib	315 ± 38.0 (3)	38.2 ± 4 4 (3)
fasted (one day)	343 ± 126 (3)	37.2 ± 22.0 (3)
Hypoxic (one day)	367 ± 177 (3)	32.5 ± 7 4 (3)
Control		
fed ad lib	494 ± 144 (5)	41 6 ± 10.9 (5)
pair-fed (seven days)	339 ± 8.3 (3)	29.8 ± 10.9 (3)
Hypoxic (seven days)	286 ± 114 (8)[a]	30.5 ± 6.7 (8)[b]

a = $p < 0.05$ as compared with the *ad lib.* control values of the same group.
b = $p < 0.1$ as compared with the *ad lib* control values of the same group.

Discussion

The results demonstrate a significant adaptation of the energy metabolism to hypox the cellular level in one day when evaluated from the redox state in the cytosol and mi chondria. The partial recovery of the initially high lactate/pyruvate ratios and lact concentrations (data not shown) during the adaptation period of seven days probab indicate diminished anaerobic glycolysis during the adaptation period. However no sim taneous decrease in ATP content occurred, and the phosphorylation state increased dur the adaptation period.

The slight increase in the ATP concentration concomitantly with the decrease in t lactate/pyruvate ratio during adaptation may be due to rapid mechanisms used to impr oxygen transport to the tissues or to reduce the consumption of ATP itself Adaptive mech nisms which may be of significance in elevating the tissue oxygen concentration within a day include an increased hepatic artery blood flow (Fischer *et al* 1960) and changes in t oxygen-carrying capacity of the blood mediated by increased erytropoietin synthesis (Syn sen and Harris 1973), a rapid relase of reticulocytes into the blood stream by erytropoie (Fruhman and Fischer 1962) and an increase in the red cell 2,3-diphosphoglycerate concent tion (Lenfant *et al* 1968) (Na –K)-stimulated ATP-ase probably contributes significa to the total ATP consumption of hepatic tissue, as has been demonstrated at least af prolonged ethanol administration (Bernstein *et al* 1973) and thyroxine treatment (Is *et al* 1973) A decrease in (Na –K)-stimulated ATP-ase activity in anoxic isolated perfu heart has been reported in one study (Balasubramanian *et al* 1973) However the pres results suggest that mechanisms other than the modulation of the activity of (Na –K stimulated ATP-ase are responsible for the adaptation of the cellular ATP consumption hypoxia.

Inhibition of the uptake of ¹³¹I by the thyroid gland, the concentration of thyroxine the gland and the secretion of iodine by the gland within a few days in hypoxic hypoxia h been observed by Galton (1972) In the present case a slight inhibition of thyroid funct

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The effect of vigorous physical activity at work on serum lipids with a special reference to serum high-density lipoprotein cholesterol

By

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Abstract

HTONEN, A. and J. VIIKARI. *The effect of vigorous physical activity at work on serum lipids ith a special reference to serum high-density lipoprotein cholesterol.* Acta physiol. scand. 1978. *104.* 117–121.

rum lipid concentrations of lumberjacks whose occupational physical activity is most vigorous were mpared with those of electricians. The lumberjacks had significantly higher serum HDL-cholesterol and nificantly lower triglyceride and free fatty acid concentrations but there were no significant differences total cholesterol levels. The favourable effect of vigorous physical activity at work on lipid metabolism however, epidemiologically not seen, obviously due to negative risk factors in lumberjacks' life.

y words: cholesterol, high density lipoproteins, serum; physical activity; work activity

he distribution of plasma lipoprotein cholesterol may be influenced by the level of habitual hysical activity in leisure time so that very active persons have higher high-density lipoprotein (HDL) levels (Carlson and Mansfeldt 1964, Lopez *et al.* 1974, Wood *et al.* 1976), ut any reports about the effect of vigorous physical activity at work on the HDL-cholesterol ave not been published. High concentration of HDL may be protective against coronary heart disease (CHD) to some degree (Nikkilä 1953, Carlson and Ericsson 1975, Berg *et al.* 1976). Prospective studies have shown an increased incidence of ischemic heart disease with even mildly raised serum levels of cholesterol (Truett *et al.* 1967) and triglycerides (Carlson and Böttiger 1972). We have compared the serum lipid concentrations of lumberjacks whose occupational physical activity is most vigorous with those of a group of ordinary electricians.

Material and methods

Subjects. All subjects completed a questionnaire on their normal diets, on smoking habits and on alcohol intake habits.

Lumberjacks (Group A, 12): Included in this group were only men who had worked at least two years as full-time lumberjacks and who were clinically healthy.

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The effect of vigorous physical activity at work on serum lipids with a special reference to serum high-density lipoprotein cholesterol

By

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Abstract

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Serum lipid concentrations of lumberjacks whose occupational physical activity is most vigorous were compared with those of electricians. The lumberjacks had significantly higher serum HDL-cholesterol and significantly lower triglyceride and free fatty acid concentrations but there were no significant differences in total cholesterol levels. The favourable effect of vigorous physical activity at work on lipid metabolism is, however, epidemiologically not seen, obviously due to negative risk factors in lumberjacks' life.

Key words: cholesterol, high density lipoproteins, serum physical activity, work activity

The distribution of plasma lipoprotein cholesterol may be influenced by the level of habitual physical activity in leisure time so that very active persons have higher high-density lipoprotein (HDL) levels (Carlson and Mossfeldt 1964, Lopez *et al.* 1974, Wood *et al.* 1976), but any reports about the effect of vigorous physical activity at work on the HDL-cholesterol have not been published. High concentration of HDL may be protective against coronary heart disease (CHD) to some degree (Nikkilä 1953, Carlson and Ericsson 1975, Berg *et al.* 1976). Prospective studies have shown an increased incidence of ischemic heart disease with even mildly raised serum levels of cholesterol (Truett *et al.* 1967) and triglycerides (Carlson and Böttiger 1972). We have compared the serum lipid concentrations of lumberjacks whose occupational physical activity is most vigorous with those of a group of ordinary electricians.

Material and methods

Subjects. All subjects completed a questionnaire on their normal diets, on smoking habits and on alcohol intake habits.

Lumberjacks (Group A, n = 12): Included in this group were only men who had worked at least two years as full-time lumberjacks and who were clinically healthy.

TABLE I. Age and physique of lumberjacks and electricians.

Group	Age (years)	Weight (kg)	Height (cm)	Weight / Height − 100
A				
lumberjacks n = 12	42.4 ± 12.3 (25–57)	76.0 ± 15.8 (6.–120)	172.7 ± 4.8 (165–181)	1.04 ± 0.17 (0.92 1.48)
B				
electricians n = 15	46.5 ± 7.6 (33–58)	83.0 ± 9.1 (69–101)	179.3 ± 6.5 (166–188)	1.05 ± 0.09 (0.95–1.21)

Electricians (Group B, n = 15). Their occupational physical activity was low or moderate and they w without regular leisure time exercise.

The age, weight height and relative weight of the lumberjacks and the electricians are shown in Tabl 7/12 lumberjacks and 5/15 electricians were smokers. All had normal diet and alcohol consumption w low or moderate. The mean working time of the lumberjacks in this occupation was 13.9 years.

Chemical methods. Venous blood was drawn after fasting overnight 12 16 h before the test. All l worked normally on the previous day. Plasma was separated by low speed centrifugation. Low den (LDL) and very low density lipoproteins (VLDL) were precipitated with a polyethylene-glycol solut (PEG-6000, final concentration 12 %) and HDL was determined from the supernatant as described ea (Viikari 1976). Cholesterol was measured by a modification of the method of Badzio and Boczo (19 Triglycerides were measured by the method of Carlson (1963). Free fatty acids were determined accord to Mikač Devic (1973).

Results

Plasma lipid concentrations in each group are shown in Table II. Plasma total choleste concentrations were somewhat higher in the lumberjacks, but this difference was not stat tically significant. This was obviously due to the higher HDL-cholesterol concentrati ($p < 0.001$) since there was no difference in (VLDL + LDL)-cholesterol values. The cumul tive distributions of HDL-cholesterol values are shown in Fig. 1. Three lumberjacks w overweight (relative weights 1.16, 1.25 1.48) had the lowest HDL-cholesterol concent tions (59.1 59.6, 62.1 mg/100 ml). HDL-cholesterol levels of other lumberjacks were clea

TABLE II. Mean plasma lipid and free fatty acid concentrations.

Group	Total cholesterol mmol/l	HDL cholesterol mmol/l	(VLDL + LDL)-cholesterol mmol/l	Triglycerides mmol/l	Free fat acid mmol/l
A lumberjacks	5.79 ± 0.83	1.93 ± 0.28	3.86 ± 0.87	0.60 ± 0..4	0.39 ± 0
B electricians	5.20 ± 1.21	1.42 ± 0.31	3.78 ± 1.23	0.92 ± 0.41	0.51 ± 0
Statistical difference (t-test) between A and B	n.s.	$p < 0.001$	n.s.		



1. Cumulative distribution of HDL-sterol. Group A (lumberjacks), B (electricians). overweight (kg/ 100>1 10); O, alcohol consumption is moderate.

er than normal. There was no correlation between alcohol intake habits and HDL-esterol. There were two mildly hypercholesterolemic men in group A (serum cholesterol and 7.5 mmol/l). Plasma triglyceride level of the lumberjacks was lower than that of the tricians. Free fatty acid concentrations were lower in lumberjacks as well.

Discussion

re are many studies concerning the effect of physical exercise in leisure time on serum ds. The influence of physical exercise on serum triglyceride level has been consistently ourable (Holloszy *et al.* 1964, Hurter *et al.* 1972, Wood *et al.* 1976). Plasma triglyceride centration and age are positively correlated in sedentary subjects but not in athletes rter *et al.* 1972). A program of vigorous exercise has been followed by modest reduction serum cholesterol concentration (Kilbom *et al.* 1962, Mann *et al.* 1969 Wood *et al.* 1976), ile in other studies no such change was observed (Holloszy *et al.* 1964). There are only v reports concerning the effect of physical activity at work on serum lipids. No differences serum cholesterol and triglyceride concentrations were found in different occupation oups (Carlson and Lindstedt 1968). The persons with high physical activity in leisure ne have higher serum HDL-cholesterol concentrations than normal persons (Carlson and lanfeldt 1964, Lopez *et al.* 1974 Wood *et al.* 1976). This finding is prominent only after a onsiderable amount of regular and vigorous exercise. The concentration of HDL-cholesterol f the runners was clearly higher than in a group with normal physical activity when the mount of kilometres run was more than 70 km per week (Lehtonen and Viikari, in press). Runners expended an average of about 5 000 kcal/week more energy than physically in-ctive controls. Our group of lumberjacks was small, but serum HDL-cholesterol concentra-tions in this group were significantly higher than in physically inactive men. The work of umberjacks is known to require more energy than any other occupation. Recorded energy expenditure figures range from 4 500 to 8 000 kcal per day (Karvonen and Turpeinen 1954 Karvonen et al. 1961).

According to our results the effect of vigorous work activity on serum HDL-cholesterol cerides is similar to that of exercise in leisure time. There were no major differences

in the alcohol intake habits of these two groups that would explain the difference. In group of lumberjacks three men were obese and their HDL-cholesterol levels we lowest in the group but triglyceride concentrations were normal. Total cholesterol conce tions in lumberjacks were slightly higher than in electricians. This was obviously du higher HDL-cholesterol Free fatty acid levels were slightly lower in lumberjacks. The with high physical activity in leisure time had normal serum cholesterol levels but hi free fatty acid concentrations (Lehtonen and Viikari, in press).

Many reports have supported the idea that relatively low levels of plasma HDL-chole are associated with an increased risk of coronary heart disease (Nikkilä 1953 Castelli 1975 Carlson and Ericsson 1975 Berg *et al* 1976) and an increased incidence of isch heart disease with even mildly raised serum levels of cholesterol (Truett *et al* 1967) triglycerides (Carlson and Böttiger 1972) has been shown. There is, however an in relationship between VLDL-triglycerides and HDL-cholesterol as summarized by M and Miller (1975) so that in fact the decrease of serum triglycerides and the increa serum HDL-cholesterol may reflect the same phenomen

The effect of increased physical work activity on serum HDL-cholesterol and triglyc is advantageous as evidenced by the results of this study If extreme physical work ac prevents the development of coronary heart disease, the evidence could be found amon lumberjacks. However the lumberjacks who were initially free of coronary heart di did not manage better but rather worse as regards the development of coronary heart di than men with a less heavy occupation on the 10-year experience (Punsar and Karv 1976) This might be because other risk factors may promote the development of coro heart disease in lumberjacks. Smoking is a risk factor of major importance and ther more smokers in the lumberjacks than in control occupational groups (Karvonen 1961) The effect of lower socioeconomic status and working in difficult circumstance be deleterious. On the other hand, longshoremen with heavy work activity had a l death rate from coronary heart disease than their counterparts (Paffenberger and Hale In Rissanen's (1976) study the favourable effect of physical work activity was also Men in occupations demanding regular physical activity had less extensive involveme coronary arteries with atherosclerosis than men in sedentary occupations. As a conc it was found in this small material that vigorous activity at work has a beneficial effe lipid metabolism.

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in the alcohol intake habits of these two groups that would explain the difference. In the group of lumberjacks three men were obese and their HDL-cholesterol levels were lowest in the group but triglyceride concentrations were normal. Total cholesterol concentrations in lumberjacks were slightly higher than in electricians. This was obviously due to higher HDL-cholesterol. Free fatty acid levels were slightly lower in lumberjacks. The ... with high physical activity in leisure time had normal serum cholesterol levels but ... free fatty acid concentrations (Lehtonen and Viikari, in press).

Many reports have supported the idea that relatively low levels of plasma HDL-cholesterol are associated with an increased risk of coronary heart disease (Nikkilä 1953, Castelli et ... 1975, Carlson and Ericsson 1975, Berg *et al.* 1976) and an increased incidence of ischaemic heart disease with even mildly raised serum levels of cholesterol (Truett *et al.* 1967) ... triglycerides (Carlson and Böttiger 1972) has been shown. There is, however, an inverse relationship between VLDL-triglycerides and HDL-cholesterol as summarized by ... and Miller (1975) so that in fact the decrease of serum triglycerides and the increase ... serum HDL-cholesterol may reflect the same phenomenon.

The effect of increased physical work activity on serum HDL-cholesterol and triglycerides is advantageous as evidenced by the results of this study. If extreme physical work activity prevents the development of coronary heart disease, the evidence could be found among lumberjacks. However, the lumberjacks who were initially free of coronary heart disease did not manage better but rather worse as regards the development of coronary heart disease than men with a less heavy occupation on the 10-year experience (Punsar and Karvonen 1976). This might be because other risk factors may promote the development of coronary heart disease in lumberjacks. Smoking is a risk factor of major importance and there ... more smokers in the lumberjacks than in control occupational groups (Karvonen et ... 1961). The effect of lower socioeconomic status and working in difficult circumstances ... be deleterious. On the other hand, longshoremen with heavy work activity had a low ... death rate from coronary heart disease than their counterparts (Paffenberger and Hale 19... In Rissanen's (1976) study the favourable effect of physical work activity was also ... Men in occupations demanding regular physical activity had less extensive involvement ... coronary arteries with atherosclerosis than men in sedentary occupations. As a conclusion it was found in this small material that vigorous activity at work has a beneficial effect ... lipid metabolism.

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From the Institute of Medical Biology University of Tromsø, Norway

Blood flow, calcium deposition and heat loss in reindeer antlers

By

KNUT HOVE* and JOHAN B. STEEN

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Abstract

HOVE, K. and J B. STEEN *Blood flow calcium deposition and heat loss in reindeer ant*
Acta physiol scand. 1978 *104* 122–128

Antler blood flow was studied in a 2 year old male reindeer during the last half of the antler growth p using an electromagnetic flow probe chronically implanted around the superficial temporal artery Arter venous (a-v) differences of calcium were measured on antler blood. The blood flow increased from 8 ml/min when the antler was half-grown to 100–120 ml/min when fully developed. Subsequently a reduc was observed towards shedding. Positive a-v plasma calcium differences (on average 0.2 mM) were re corded during the period of active growth. Two bulls maintained positive a-v calcium differences d a 48 hour starvation period in spite of reduced arterial calcium concentrations. Exercise to near exhaus caused a 2°C rise in the rectal temperature. Antler blood flow was decreased immediately after exercise returned to pre-exercise values usually within 5–10 min. Since no overshoot in antler blood flow recorded during the hyperthermia it is concluded that variations in blood perfusion of the antlers are out importance in the defence against hyperthermia during and after e ercise

In Northern Norway reindeer antlers begin to grow in March and are usually fully develo by the beginning of July During the growth period the antlers are covered by a hig vascularized, velveted skin This skin remains vascular until August when it is shed, expos the bony brown-grey antler cores. During the mating season and winter the antlers important intraspecies symbols of social rank (Espmark 1964 Henshaw 1969). The ma shed their antlers in mid winter the females a few months later This delay enables t females, privileged by the presence of antlers, to secure food which the males have labour to expose from underneath the snow cover

Apart from this social aspect of antler function it has also been suggested that the va cularized antler skin has a function in defense against hyperthermia during the summ season (Stonehouse 1968) Direct evidence supporting this suggestion seems to be lacki and the proposal of Stonehouse (1968) has been seriously questioned on behavioural a phylogenetic grounds by Henshaw (1969). Wika and Krog (1971) and Wika *et al* (197 have also questioned the role of antlers in heat dissipation after measurements of surfa temperatures under different physiological situations. In the present study in order to obta

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1 Position of flowprobe and leads on the superficial temporal y in the animal used for blood flow measurements.

e direct evidence as to the role of antlers in thermoregulation we have measured blood through the antlers and antler temperatures before and after exercise hyperthermia.
he bony antler has approximately the same mineral composition as ordinary skeletal e, and the shedding of the antlers represents a considerable loss of minerals from the ly Insufficient nutrition is known to retard antler growth (Bubenik 1966). To study deposition of calcium in antler bone we have measured the extraction of calcium from od during maintenance feeding and in starved animals.

Material and methods

[illegible]. The study was carried out on two domesticated, 2-year old male reindeer (*Rangifer tarandus [illegible]*) of very gentle disposition. The animals were kept in 4 4 m indoor pens on light regime about days in advance of normal. On May 1st they were moved to an outdoor area. They were fed pelleted concentrate [illegible] and occasionally given supplements of birch branches, hay and grass.
[illegible] loops were established in both animals month before measurements started. The animals were [illegible] with sodium pentobarbital and intubated. The carotid artery was exposed and surgically [illegible] in a permanent skin loop on the throat. The loops have orked satisfactorily for more than year [illegible] of repeated punctures.
[illegible] blood flow. An electromagnetic Nycotron blood flow probe (*I.D.* = 3 mm) was fastened on the [illegible] temporal artery in one of the bulls [illegible] half grown antlers as shown in Fig. 1. The bull was [illegible] and local anesthesia infiltrated subcutaneously in the implantation area. The artery to the right [illegible] was exposed through 10 cm incision and dissected free from connective tissue. Several side branches [illegible] supply the skin of the skull were ligated and the flow probe positioned around the artery just above [illegible] cheek bone. The probe was secured to surrounding connective tissue and the lead tunneled under the [illegible] and taken out through small incision behind the antlers (Fig. 1). Local antibiotic application at the [illegible] exit temporary prevented infection and insect repellent prevented disturbance from flies. Despite [illegible] precautions local infection forced us to remove the probe on *July* 23.
For measurements of blood flow the probe was connected to Nycotron recorder. Zero flow was ob- [illegible] by compressing the artery here this traverses the cheek bone. T control that all side branches had [illegible] tied off, *i.e.* that the recorded blood flow represented the total flow through one antler, we checked [illegible] flow was also zero hen compression was applied by hand around the base of the antler. In vivo [illegible] of the flow signal could not be performed, therefore the calibration factor offered by the manu- [illegible] was used to obtain actual flow values.
[illegible] flow was recorded at weekly intervals from April 20, to June 25

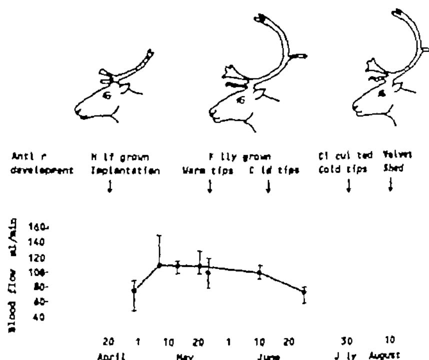


Fig. 2. Antler development and changes in antler blood flow (average and range of observations in a 20–60 min period of measurements). Blood flow after 48 hours of starvation.

Blood samples were taken at about one week intervals from both animals. Arterial blood was o[illegible] from the carotid loop. Venous blood was drawn from a catheter positioned in a vein at the base of antler. Due to the gentle disposition of our animals the samples were obtained with a minimum of [illegible] motion and without the use of drugs. Plasma calcium was measured by atomic absorption spectrophotometry.

Body temperature (rectal) was measured at a 10 cm depth by an electronic thermometer. The temperature of the antler surface was measured by a thermistor covered with foam rubber during the last part of period of active growth (May 12) and near the time of velvet shedding (July 30).

Experimental

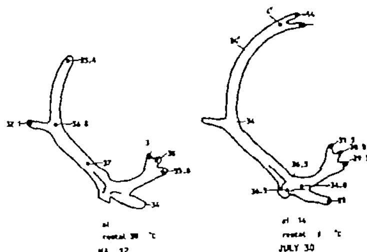
Antler development and blood flow were recorded at weekly intervals from one week after the flow [illegible] had been positioned (April 20) until June 24. Care was taken to handle the animals as gently as po[illegible] as both emotional stress and mechanical irritation of the growing antlers would decrease blood flow. B[illegible] flow was measured repeatedly at each session. Arterial and venous blood was taken at the end of [illegible] measurements.

The animals were starved, but received water, for 48 hours beginning May 20. At the end of this p[illegible] antler blood flow and arteriovenous blood samples were taken.

Temperature, flow measurements and heart rate were recorded before and after the animal had [illegible] exercised to near physical exhaustion. The exercise was expertedly conducted by a native Lapp "v[illegible] (reindeer handler) using traditional harness and sledge.

Results

The antler from which blood flow was monitored developed [illegible] extent as the contralateral control antler. Fully developed



3 Antler surface temperatures measured at rest in the period of active growth (May 12) and p- ... mately 10 days before velvet shedding (July 30).

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...imal no.	1	2
...erial	2.70 (2.55–2.78)	2.76 (2.68–2.81)
...V ...fference	0.29 (0.06–0.54)	0.14 (0.08–0.18)
...erial ...terial	2.29 (2.24–2.37)	2.47 (2.41–2.56)
...V ...fference	0.16 (+0.05–0.37)	0.20 (0.13–0.22)

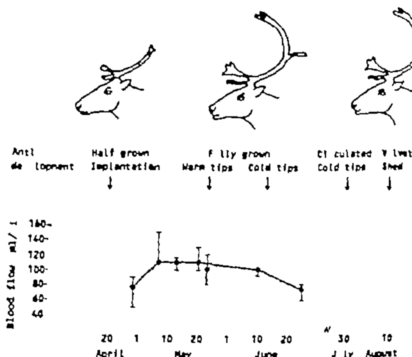


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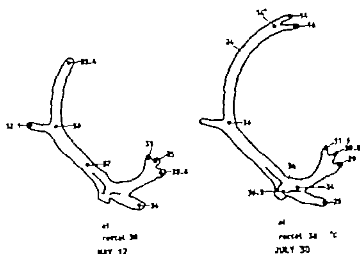
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Antler blood flow and development are shown in Fig. 2. During each recording session we obtained a range of flow values. This probably does not reflect a truly normal situation since we frequently experienced that antler flow decreased if the animal was scared or if the antlers were touched. Although we tried to avoid this, we believe that the highest values are the most representative for a normal antler flow. When the antlers were half grown the flow was 40–90 ml/min, when they were fully developed the flow was 110 to 160. This flow was maintained for the following two weeks while antler growth rate declined, and decreased thereafter. Due to infection we were unable to measure blood flow in the antlers close to the

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Animal no.	1	2
Fed		
Arterial	2.70 (2.33–2.78)	2.76 (2.68–2.81)
A–V difference	0.29 (0.06–0.50)	0.14 (0.08–0.18)
Starved		
Arterial	2.29 (2.24–2.37)	2.47 (2.41–2.50)
A–V difference	0.16 (−0.03–0.37)	0.20 (0.18–0.22)

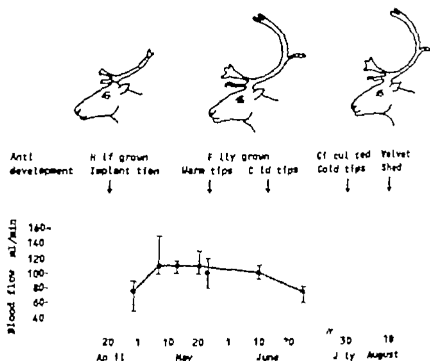


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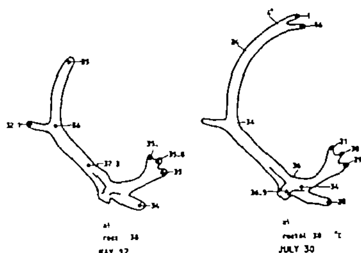
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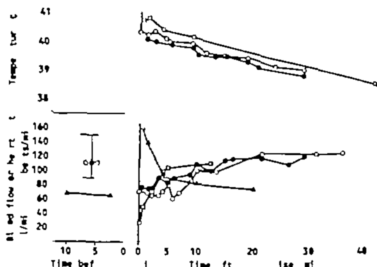


Fig. 4 Changes in rectal temperatures and blood flow during experiments with heavy exercise different occasions (□ ○ ●). Blood flow in pre-exercise period: Range and approximate recorded flows during 10 min period. The heart rate (–Δ–) was recorded manually by palpation carotid loops in one of the experiments.

time of shedding, but the low surface temperatures recorded on July 30, particularly in tips, clearly indicate that a further reduction in flow rate occurred towards shedding t (Fig. 3) The skin at the antler tips was circulated, but dry almost parchmentlike at this t

The arterio-venous Ca differences varied considerably (Table I) with an average u of about 0.2 mM Arterial plasma calcium levels were lowered after the 48 h of starvat but positive arterio-venous Ca differences were maintained in all pairs of samples ex one (Table I)

The animal with flow measurement facilities was exercised on 3 occasions between Ju and 15 when the antlers were almost fully grown. Fig. 4 shows heart rate, body tempera and antler blood flow before and after exercise at ambient temperatures between –5 r +5°C. A positive heat load is demonstrated by the profound post exercise hyperther (from normal 38.5 to about 40°C) The heart rate was 60–70 BPM at rest and about immediately after exercise. The antler blood flow was from 30 to 70% of normal immedia after termination of exercise and gradually reached normal values during the following h hour Values significantly above the pre-exercise values were only occasionally record The surface temperatures of the antlers generally increased in proportion to the bo temperature (about 2 C) when measured during the first 5 min after the exercise.

Discussion

Reindeer antlers begin as buds and grow into stalks which again divide by budding result in antlers with several distinct branches. Antler growth takes place primarily at the ti The tips have a rich blood supply and show a high radiant temperature which can be ea felt by hand or visualized by thermovision (Wika and Krog 1971). The present results indic that blood flow increased in proportion to the number of growth sites. A high flow ra

ned for a considerable time after actual length-growth has ceased. This may be as-
ated with thickness growth or antler ossification. With the approach of antler skin
dding, blood flow decreases, as is shown by direct measurements and indicated by meas-
ments of surface temperatures 10 days before shedding.

Based on our measurements of blood flow and arteriovenous calcium gradients we can
imate the amount of calcium deposited during the approximately 100 days of growth.
low of 100 ml/min and a gradient of about 0.20 mM give 90–100 g of calcium deposited.
out 17% of the dry weight of cervid antlers is calcium (Bubenick 1966, Gelbke 1973,
nneberg 1968). Our animal had an antler which weighed 900 grams indicating a total
cium deposit of 150 g. This compares favourably with our calculation and supports our
tement that the highest rather than the average recorded blood flow is normal. Alter
tively the a-v differences might have been somewhat higher in the period prior to our
asurements. The extraction of calcium by growing antlers is high enough to enable meas-
ment, and cervid antlers might therefore be a valuable organ model for the study of
lcium metabolism in rapidly developing bony tissue.

After a 48 h fast the calcium absorption from the digestive tract is probably reduced to a
nimum and the plasma calcium concentration most likely maintained by bone resorption.
10–20 per cent reduction in arterial calcium levels at the end of the starvation period clearly
licated a limited calcium supply but the levels were still well within the normal range in
ndeer (Bjarghov *et al.* 1976, Hyvärinen *et al.* 1977). Irrespective of this decrease in plasma
lcium concentrations positive arteriovenous differences across the antlers were maintained
both animals, indicating transport of calcium from skeleton to antlers during such condi-
ns. *Long term starvation or insufficient feeding is known to reduce antler growth* (Bu-
nik 1966), but it is not known if Ca shortage is the main limiting factor under such
nditions.

When reindeer are exercised to hyperthermia, heat dissipation is increased by panting
d eventually eating of snow. If heat dissipation by the antlers is of functional significance
sodilation and increased perfusion should be the result of positive heat loading. The
esent experiments demonstrated to the contrary that antler blood flow decreased as the
imal approached thermal collapse, and remained reduced in the early phases of recovery.
art rate and antler flow returned to preexercise levels within five to 20 min after the ter
nation of work, while 30–40 min were needed to dissipate the heat load incurred during
a work period. During the last 20–30 min of the hyperthermia period no overshoot in
tler blood flow was recorded. Recently Wika and Edwinsson (1978) reported evidence
the presence of adrenergic receptors in the antler arteries, and presence of well innervated
ssels below the antler. The pronounced decrease in antler blood flow observed immediately
ter the end of the exercise can thus be explained as part of the increase in vasoconstrictor
ne to skin during heavy exercise. Taylor (1966) found that the circulation through the
rns in goats may be used to regulate brain temperature by heat exchange between cool
rnual venous blood in the cavernous sinus and arterial blood in the carotid rete. The
rotid rete is present in most artiodactyls. The venous structures necessary for this heat
change also seem to be present in reindeer since we found a well developed communication
the base of the antler between the superficial temporal vein and an orbital vein leading

towards the cavernous sinus. A similar venous arrangement has been reported in Jap deer (Ohtaishi and Too 1974) Actively growing antlers have warm tips and the skin temp ture, and therefore probably also venous temperature, on the branches and stems h je few degrees below body temperature even at ambient temperatures around 0°C (Fig It therefore seems doubtful whether cooling of arterial blood to the brain by antler ve blood can be of functional importance during the period of growth, when the growth p cesses probably determines the flow This might be different when the antlers are L developed, however since the surface temperatures then approach ambient tempera (Fig. 3) and cooled blood is returned to the head. In conclusion, the results of the pre study demonstrate that antlers do not function as radiators for surplus heat during t exercise hyperthermia. It seems doubtful whether antler venous blood can serve as a coo medium for arterial blood in the carotid rete when antlers are in active growth, but it r do so when the antler is fully developed

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Isometric and dynamic endurance as a function of age and skeletal muscle characteristics

By

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Abstract

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The aim of the study was to investigate the influence of age on endurance of human skeletal muscle. An attempt was made to correlate muscular performance at various ages with some morphological and enzymatic characteristics of the muscle. Fifty healthy men, 22–65 years of age, with low daily physical activity (clerks) volunteered for the study. Isometric and dynamic endurance were determined under standardized conditions and measured in relation to maximum strength, thereby correcting for individual as well as age differences in maximum strength. Biopsies taken from the quadriceps muscle were used for muscle fibre identification, fibre area determinations, and measurements of some enzyme activities (Mg^{++} stimulated ATPase, myokinase (MK), lactate dehydrogenase (LDH), LDH isozymes). Maximum isometric and dynamic strength decreased in the older groups while no significant change was seen in isometric or dynamic endurance. Significant correlations were observed between endurance and fibre type distribution, fibre area, and LDH isozyme activities.

Key words: Fibre area, fibre type distribution, human skeletal muscle, isokinetic, lactate dehydrogenase.

Several studies have shown that maximum isometric strength of skeletal muscle decreases with age (Quetelet 1836, Ufland 1933, Asmussen and Heebøll-Nielsen 1961). The effect of increasing age on muscular endurance is, on the other hand, less well documented. Petrofsky and Lind (1975 a, b) found that isometric endurance measured as performance time at 40% of maximum isometric strength (MIS) increased with age and suggested that this was due to a change in proportions between type I and type II fibres towards a higher proportion of type I fibres. This suggestion was made since type I (slow twitch) are generally held to be more fatigue-resistant than type II (fast twitch) fibres. Type II fibres are, on the other hand, associated with greater strength and higher speed of movements (for ref. see Burke and Edgerton 1975).

Hulkko et al. (1975), studying the properties of the quadriceps muscle in a group of young men, have shown that a correlation exists between isometric endurance and percentage of type I fibres. Also dynamic endurance, measured as the ability to maintain tension output during repeated dynamic knee extensions, was found to correlate with the histochemical

properties of the quadriceps muscle (Thorstensson and Karlsson 1976). More recent stud have shown that lactate accumulation total lactate dehydrogenase (LDH) as well as LD isozyme activities are of importance for dynamic endurance in the contracting quadric muscle (Tesch *et al* 1978)

The results of the studies cited above thus suggest that the histochemical and biochemic properties of muscle are important factors for an individual s capacity to perform lon lasting isometric or repeated dynamic contractions. The aim of the present study was t test this hypothesis by measuring muscular endurance in various age groups of a populatie of sedentary males, for which the relation between age and fibre type proportions and enzyr properties had already been determined (Larsson *et al* 1978 a) Since the quadriceps mus was used for these histochemical and biochemical studies, this muscle was also used for t studies of mechanical performance. The choice of the quadriceps muscle had an additio advantage because it has been shown that ageing processes, such as decline in maximu strength and muscle atrophy occur relatively early in this muscle (Charles 1964 Serrate *et al* 1968 Tomlinson *et al* 1969)

Material and methods

50 healthy male subjects from 22 to 65 years of age volunteered for the study The subjects were employ of an insurance company (clerks) and were in general minimally physically active, and all could be cl sified in groups I–II according to Saltin nd Grimby s (1968) classification of occupational and sparetime physical activity On physical examination they were found to be without locomotory defects.

Height, weight, skinfold thickness, and sk letal breadth (femoral condylar and bistyloid radioulna were measured for each subject to estimate f t free soft tissue weight (FFS) by the method of Herman and von Dobeln (1971).

Maximum isometric strength (MIS) and isometric endurance, respectively were determined for bo legs simultaneously (Karlsson and Ollander 1972) by recording the force exerted when the subjects pres their feet against a stiff bar equipped with a force transducer MIS was taken as the highest force va obtained during series of five contractions. After three minutes rest isometric endurance time was r corded, measured as the maximum time during which a tension level of 50 % of MIS could be maintain

Maximum dynamic strength and dynamic endurance were measured with a commercial isokin dynamometer (Cybex II Lumex Inc., New York) in the left knee extensor muscles at a preset veloc of π rad/s (180 deg/s). The subject was sitting in a experimental chair with hip angle of π rad (90 degre The lower leg moved the lever of the dynamometer the knee joi t being al gned with the fulcrum of t lever The subject was instructed to e ert maximum force against the lever through its whole angular mov ment from 0 55 π to 0 rad (100 to 0 degrees, *i e* full knee xtension). Maximum dynamic strength (MD was defined as the highest value for peak torque from two knee extensions. Dynamic endurance was c culated from measuring 50 maximal contractions and determining the bsolut (Nm) or relative (%) cline in peak torque from the mean of the three initial contractions to the mean f the three final contr tions. Thus, dynamic ndurance increased with decreas g bsolut or relative decline. For detailed formation of the apparatus and experimental set-up, see Thorstensson (1976), Thorstensson and Karlss (1976), and Thorstensson *et al* (1976).

The subjects in this paper are identical with those employed for two previous investigations of maxim strength, muscle histochemistry and enzyme activities t various ges (Larsso *et al* 1978 a, b) except f five men who were unable to participate here Since the results of the previous study are discussed here a used for correlation with the mechanical performance data, a brief account f the methods used is giv

Two muscle biopsies were taken from the vastus lateralis muscle (Bergström 1962), one was used f histochemical analyses, the other for enzyme activity analyses. In all subjects, the muscle f bres were cl sified into type I (slow twitch) and type II (fast twitch) (Engel 1962). The verage cross-sectional area of t two main f bre types as well as the relative cross-sectional rea of each f bre type in the muscle were det mined (Gollnick *et al* 1972, Dubowitz and Brook 1973). I 40 of the subjects the type II fibres

TABLE I Height, body weight, and fat free soft tissue weight (FFS) of the different age groups.

Age group (yrs)		Average age (yrs)	Height (cm)	Body weight (kg)	FFS (kg)
20–29	10	26.1±0.8	182.9±2.0	75.8±3.2	53.9±2.4
30–39	10	35.3±1.0	180.2±2.1	78.1±3.1	54.1±2.6
40–49	8	42.6±0.8	181.9±1.3	77.6±2.8	54.6±2.0
50–59	12	54.5±0.6	179.8±1.3	79.2±1.4	54.1±1.4
60–65	10	61.6±0.6	176.4±2.5	75.2±3.3	52.4±2.4

Values are means ± S.E.

classified into A, B, and C subgroups (Brooke and Kaiser 1970, Dubowitz and Brooke 1973). The activities of Mg^{2+} stimulated ATPase, myokinase (MK), and lactate dehydrogenase (LDH) were determined fluorometrically (Lowry and Passonneau 1972). The LDH isozyme pattern was quantified by means of densitometric scanning (Pearsey et al. 1974).

Step wise linear multiple regression analyses have been performed. For data processing, the SPSS (Statistical package for the social sciences) (Nie et al. 1975) was used with MIS, MDS, isometric endurance and absolute or relative decline in dynamic strength as dependent variables. Independent variables were the anthropometric, histochemical, and biochemical variables together with subject's age. Pair wise deletion of missing data was used (Options 2, Nie et al. 1975). In each step in the analysis, the variable added to the regression equation is the one which makes the greatest reduction in the error sum of squares of the dependent variable and has the highest partial correlation with the dependent variable in proportion to the variables which have already been added to the equation. The last step (equation) given in Results is the step with minimum residual variance.

Arithmetic means and standard errors were also calculated. The significance of linear correlation coefficients was tested from individual values according to Snedecor and Cochran (1967).

Results

Anthropometric data in the different age groups are presented in Table I. A comparison of these groups with representative age-matched groups of the Swedish male population revealed a significant difference ($p < 0.05$) only in height in the 50–59-year-old groups, *i.e.* the subjects presented here were taller compared with 50–59-year-old representative Swedish males (179.8 cm and 174.3 cm, Levin, personal communication).

Summary of histochemical and biochemical results (Table II). The proportion of type II fibres decreased with age. Type II fibre subclassification revealed a decrease in percentage

TABLE II Fibre type distribution, fibre area ratios, and LDH isozyme activities in the muscle of the different age groups.

Age group (yrs)		Average age (yrs)	Fibre type distribution (% type I)	Fibre area ratio (type II/I area ratio)	LDH isozyme activities (µmoles g⁻¹ min⁻¹) M	H
20–29	10	26.1±0.8	43.0±3.1	1.30±0.08	34±5	10±1
30–39	10	35.3±1.0	36.8±1.5	1.24±0.09	46±7	17±4
40–49	8	42.6 [illegible] 0.8	48.2±5.1	1.08±0.07	30±5	17±2
50–59	12	54.5±0.6	51.7±3.0	0.99±0.05	20±3	16±3
60–65	10	61.6±0.6	55.0±4.5	0.99±0.09	23±3	16±3

Values are means ± S.E.

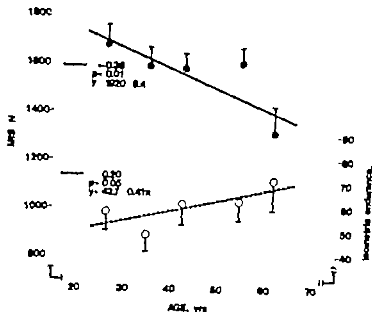


Fig. 1 Maximum isometric strength (MIS) and isometric endurance versus age. The symbols represent: ● = MIS and ○ = isometric endurance. Mean values + or − S.E. are shown along with the regression lines. Regression lines are calculated from individual values.

of both type IIA and IIB fibres during ageing, while no change was seen in percentage of IIC fibres. Fibre area determinations, however showed that the area of type I fibres remained unchanged while there was a decrease in type II fibre area. Consequently the II/I fibre area ratio and relative type II fibre area decreased from 1.30 to 0.99 and 61 to 44% in the 20–29 and 60–65-year-old group, respectively The anaerobic M LDH activity decreased during ageing while no change was seen in the "aerobic" H LDH activity resulting in a decreased M/H LDH activity ratio (Table II). The activities of Mg^{2+} stimulated ATPase and myokinase were, on the other hand, unchanged (Larsson *et al* 1978 a).

Maximum strength MIS (two-leg) and MDS (one-leg) followed a similar pattern during ageing, *i.e* a marked decline was only seen in the oldest groups (Fig. 1 and 2). The decline in MIS and MDS, seen in old age were observed to correlate significantly ($p < 0.001$) with the atrophy of type II fibres (Table III).

Endurance Isometric endurance, measured as the ability to maintain 50% of MIS tended to increase with age (Fig. 1) Also dynamic endurance, *i.e* the inverse of the absolute or relative force decline during repeated isokinetic contractions, showed a slight increase with age (Fig. 2). However the increase was not statistically significant in either case

Simple correlations Some of the histochemical and biochemical muscle characteristics previously seen to change with age (Larsson *et al* 1978 a), correlated significantly to isometric and dynamic endurance when all subjects irrespective of age were included. Isometric endurance, for instance, decreased with an increasing type II fibre area ($p < 0.05$). The relative force decline increased with an increasing proportion of type II and type IIA fibres as well as an increasing relative type II fibre area ($p < 0.05$–0.01). The absolute force decline increased with an increasing relative type II fibre area and increasing M LDH activity ($p < 0.05$–0.01).

Neither isometric endurance nor the relative force decline correlated with maximum strength, while the absolute force decline increased with an increasing MDS ($p < 0.05$

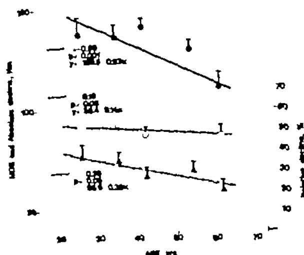


Fig. 2. Maximum dynamic strength (MDS), relative decline and absolute decline versus age. The symbols represent: ● MDS, ○ – relative decline and ▲ – absolute decline. Note that small force decline corresponds to high dynamic endurance and vice versa. Mean values ± S.E. are shown along with the regression lines. Regression lines are calculated from individual values.

Partial correlations: In simple as well as in multiple regression analyses, isometric endurance correlated significantly only with type II fibre area (Table III). The same general relationships seen in simple regression analyses for dynamic endurance were also seen in multiple regression analyses.

Discussion

Muscular endurance (isometric and dynamic) has been reported to decrease during ageing (Collumbine *et al.* 1950, Burke *et al.* 1953, Simonson 1977). However, in these studies, no correction was made for the decrease in maximum strength during ageing. The major observation in the present paper was that no decrease in muscular endurance could be seen with increasing age, when correcting for the decrease in maximum strength, if anything, endurance tended to increase.

So far few studies have demonstrated changes in isometric endurance with age. Petrofsky and Lind (1975 a, b) studied maximum handgrip strength and handgrip endurance (40% of maximum strength until the subjects could no longer maintain tension) in 100 industrial workers aged from 22 to 62 yrs. No significant changes were found in maximum strength or endurance although endurance tended to increase with age. However, body weight increased with age and when multifactor analysis was used to delineate the independent contribution of weight to strength and endurance, maximum strength decreased and endurance increased with age. The present findings are thus in agreement with these data. Petrofsky and Lind suggested, on the basis of results of animal studies (Drahota and Gutmann 1963, Close 1964, Gutmann and Hanzlíková 1972), that the changes found in maximum strength and endurance with age might be due to changing proportions of type I and type II fibres giving a higher proportion of type I fibres with age. Since such a change in fibre proportions has been observed in human muscle (Larsson *et al.* 1978 a) the hypothesis appears tenable. The negative correlation ($p < 0.05$) between isometric endurance and type II fibre area ob-

TABLE III Step wise multiple regression analyses with independent variables presented in step wise order. The simple regression coefficients with the dependent variable are also presented.

Dependent variable	Independent variables in stepwise order	Regression coefficients		Multiple correlation coefficient[a]
		simple	partial[1]	
Maximum isometric strength (MIS)	1 Type II fibre area	0.17 **	0.18**	0.51***
	2. H LDH activity	788	764	0.55***
	3 M LDH activity	440	249	0.59***
Maximum dynamic strength (MDS)	1 Age	0.93	−0.80**	0.50***
	2. FFS	1.0	0.85	0.56***
	3. Type II fibre area	0.014 **	0.0065	0.59***
Isometric endurance	1 Type II fibre area	−0.01	−0.01	0.34
	2. Myokinase activity	−0.09	−0.10	0.38
Absolute force decline	1 M LDH activity	50.7*	42.0*	0.51**
	2. FFS	1.2*	1.1	0.60**
	3 Type I fibre area	−0.004	−0.006	0.65**
	4. Myokinase activity	−0.008	−0.10	0.67**
	5. % type II fibres	0.24	0.30	0.70**
Relative force decline	1 % type II fibres	0.21	0.29*	0.38*
	2. M LDH activity	14.6	13.1	0.45
	3. Myokinase activity	−0.009	−0.05	0.53
	4 Type I fibre area	−0.002	−0.003	0.57*
	5. FFS	0.27	0.28	0.60*

* = $p < 0.05$
** = $p < 0.01$
*** = $p < 0.001$

[1] Partial regression with the dependent variable when all independent variables are included in the regression.

[a] Multiple correlation with the dependent variable when all variables up to each step are included in the regression equation.

tained here is also in line with the results of Hultén *et al* (1975) who reported a negative correlation ($p < 0.01$) between isometric endurance and per cent type II fibres.

Until now no standardized measurements of dynamic muscular endurance and its relation to age have been published. In the present study a recently described test (Thorstensson and Karlsson 1976) was used to measure dynamic endurance in which the subjects performed repeated maximum knee extensions at a high angular velocity. The peak torque was registered and the relative (per cent) and absolute (Nm) force decline could be calculated and used as expression for dynamic endurance, *i.e.* the dynamic endurance increased with decreasing relative or absolute force decline. The relative force decline in this material was found to be independent of MDS but increased with an increasing type II fibre population as earlier shown by Thorstensson and Karlsson (1976), although the correlation here was poorer.

It has been suggested that lactate accumulation (preferentially in the type II fibres) and a postulated lactate translocation from type II to type I fibres are limiting factors for dynamic endurance measured by the method used here (Tesch *et al* 1978). According to this hypothesis type I fibres would serve as a lactate recipient for lactate produced in the type II fibres. The increased dynamic endurance with an increasing relative area of the type I

...res, found in the present study supports this hypothesis. The decreased endurance with increasing M LDH activity also indicates that the produced lactate is an important ...ting factor in this type of exercise. The decline found in M LDH activity and in the ...oportion of type II fibres with age (Larsson *et al.* 1978 a) are in agreement with the tendency ...wards an increased dynamic endurance with age.

The same general relationships found between histochemical data and endurance data ...scribed by Hultén *et al.* (1975) (isometric endurance) and Thorstensson and Karlsson ...976) (dynamic endurance) have been found in the present study although the correlations ...ere poorer. This was also true when multifactor analysis was applied to delineate differences ...age, muscle mass (FFS), maximum strength etc. The poorer correlations might, however ...due to other ageing processes interacting with the muscle variables presented here, such as ...duced intramuscular blood flow (Simonson 1964), structural changes in the mitochondria ...rlander *et al.* 1978), and changes in the neuromuscular junction (Gutmann and Hanzlíková ...72). The smaller variance in fibre type distribution observed here compared with those in ...e studies by Hultén and Thorstensson and their co-workers probably also contribute to ...e poorer correlations.

In summary no decline was seen in isometric or dynamic endurance during ageing (when ...easured in relation to maximum strength). Tendencies were, on the contrary observed ...wards increased endurance with age. These tendencies are in agreement with some of the ...anges seen in the histochemical and biochemical muscle characteristics during ageing. ...owever maximum strength decreased during ageing, indicating a reduced isometric and ...ynamic endurance capacity when measured in absolute terms.

...he authors are indebted to Dr Cecil Fox for stimulating discussions of the manuscript, and to Björn ...mason and Rickard Schéle for valuable statistical advice.

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Effect of metabolic inhibitors on phenylalanine influx in cerebral cortex slices from mature and immature rats

By

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Abstract

LAAKSO, M. L. *Effect of metabolic inhibitors on phenylalanine influx in cerebral cortex slices from mature and immature rats. Acta physiol. scand. 1978 104 137 147*

…lier investigations have shown that the transport of aromatic amino acids into cerebral cortex slices of rats changes during ontogenetic development. In order to illuminate the possible influence of the developmental alterations of energy metabolism in the brain on the changes of the amino acid transport the influx of phenylalanine into cerebral cortex slices of adult and 7-day-old rats was measured in the presence of metabolic inhibitors. Also the levels of lactate, ATP ADP AMP and creatine phosphate in the slices were measured. The used inhibitors cyanide, dinitrophenol and iodoacetate, which disturb certain steps of energy metabolism, had very similar effects in both age groups on phenylalanine influx and on the levels of energy-rich phosphates. The correlation between the level of ATP and the influx of phenylalanine was positive in both age groups, but the slopes of the regression lines were different. If ATP supplies energy for amino acid transport, its utilization, rather than its availability is a limiting factor in the slices from 7-day-old rats. Developmental changes in energy metabolism seem to have a minimal role in the changes of amino acid transport during the maturation of the brain. Changes in the properties of cell membrane carriers and in the transmembrane ionic gradients may be more important.

Key words: cerebral cortex slices, transport of amino acids, metabolic inhibitors, brain development, phenylalanine

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The opinions of investigators differ as to how energy is derived for concentrative uptake of amino acids by cells. Energy-rich phosphates, ATP in particular, are thought to be involved the most often. Even if a direct coupling of the influx of amino acids with the hydrolysis of ATP has been disputed (Geck *et al.* 1974), there appears to be some kind of correlation between the rate of influx of amino acids and the intracellular level of ATP in slices of nervous tissue (Abadom and Scholefield 1962, Barbosa *et al.* 1968, Banay-Schwartz *et al.* 1971 Baker and Potashner 1973, Banay-Schwartz *et al.* 1974). This correlation is not, however consistent under all incubation conditions. Energy-rich phosphates may also participate more indirectly in transport processes, e.g. by maintaining uneven distribution ratios for alkali metal ions between the intra- and extracellular fluids. The resulting electrochemical gradients may act as driving forces for transmembrane fluxes of amino acids (Riggs *et al* 1958, Vidaver 1964 Eddy 1968). Kinetic analyses of our

earlier results on amino acid transport in brain slices have indicated e.g. that the max velocities of the influx of aromatic amino acids into brain cortex slices and the half-satura- tion concentrations of the hypothetical carrier sites at cell membranes are smaller in slices prepared from 7-day-old rats than in slices from adult rats (Vahvelainen and 1972). The nature of energy metabolism in the brain changes appreciably during on- genetic development (Berg 1971). The significance of anaerobic glycolysis (Riggs et 1958, Takagaki 1974) and the hexose monophosphate shunt (Winick 1970) is greater immature brain. Piccoli et al. (1971) did not find in the slices from rat brain developmental changes of the levels of ATP which could explain the differences of amino acid transport in their study. Because ATP may have only an indirect role in the amino acid transport, it is possible that other developmental alterations in energy metabolism participate in the ontogenetic development of the transport processes.

Now we have continued our studies by comparing phenylalanine influxes into brain cortex slices of adult and 7-day-old rats incubated in the presence of iodoacetate, dini- trophenol and cyanide, all of which are known to inhibit certain steps of cellular energy metabolism. The concentrations of energy rich phosphates and lactate in the incubated slices were also measured. The main purpose of the study was to find, if there are differences between the age groups 1) in the effects of the metabolic inhibitors on phenyl- alanine influx, or 2) in the correlation of phenylalanine influx to the levels of the measured metabolites.

Materials and methods

Chemicals

L-3-phenyl(alanine-2,3-H^3) (sp.act. 1.0 Ci/mmol) was purchased from the Radiochemical Centre, Amer- ham. The metabolic inhibitors were sodium cyanide (NaCN) (J. T. Baker Chemicals N. V. Deve- ,4 (α)-dinitrophenol (DNP) (E. Merck AG, Darmstadt) and sodium iodoacetate (NaIA) (Fluka Buchs SG).

Experimental procedure

Slices of brain cortex were prepared from adult male (180–200 g) and from 7-day-old (10–15 g) Sprague- Dawley rats at 4°C with a non-wetted tissue slicer of the Stadie-Riggs type. The thickness of the slices 0.5 mm, and one side of each slice was intact brain surface. Two slices, weighing together about 100 were used in each expt. The slices were preincubated for 30 min at 37°C with shaking (80 oscillations/ amplitude 2 cm) in a Warburg apparatus under continuous flow of pure oxygen in Erlenmeyer flasks containing 4.0 ml of Krebs-Ringer phosphate solution (pH 7.4, concentrations in mmoles/L: NaCl, KCl, 5.1; $CaCl_2$, 2.7; KH_2PO_4, 1.3; $MgSO_4$, 1.3; Na_2HPO_4/HCl-buffer, 10) (Umbreit et al. 1964) glucose (10 mmol/l) and varying concentrations of the metabolic inhibitors. The control slices were preincubated without inhibitors.

After the preincubation, 1.0 ml of the incubation solution with [^3H] phenylalanine (final concentration 1.0 mCi/l) and unlabelled phenylalanine (final concentrations 0.02–2.0 mmol/l) was added to the flasks and the incubation was continued for 5 min. The slices were then rinsed briefly on a filter paper in funnel with 5 ml of cold Krebs-Ringer phosphate solution, weighed in 1.0 ml of cold 5% trichloroacetic acid (TCA) or in 1.2 ml of cold 0.6 N perchloric acid (PCA), and homogenized and centrifuged for 30 at 10 000 g. The radioactivity of the TCA supernatants and that of the incubation solutions were determined with Packard Tri-Carb scintillation spectrometer (Bray 1960), the counting efficiency having been determined with standard samples.

The concentrations of lactate, ATP, ADP and AMP were determined from the PCA supernatants enzymatically with Biochemica Test Combinations from Boehringer Mannheim GMBH (... Hohorst 1970, Jaworek et al. 1970) and the concentration of creatine phosphate by the method of ... and Rosenberg (1952).

I. Effect of metabolic inhibitors on the influx of phenylalanine into brain cortex slices of adult and 7-day-old rats, with varying concentrations of phenylalanine in the medium.

[illegible] of rats	Conc. of phenylalanine in medium (mmol/l)	Control (mmol/kg)	NaCN 0.2 mmol/l (%)	NaCN 1.0 mmol/l (%)	DNP 0.1 mmol/l (%)	NaIA 1.0 mmol/l (%)
[illegible]lt	0.02	14.5 ± 0.6	132 ± 17	41 ± 10	61 ± 5	89 ± 14
	0.05	38.2 ± 2.0	103 ± 19	46 ± 6	59 ± 8	79 ± 7
	0.2	134.7 ± 25.5	115 ± 9	53 ± 19	55 ± 5	84 ± 5
	0.5	257.1 ± 18.1	104 ± 11	53 ± 14	68 ± 7	89 ± 10
	2.0	985.2 ± 161.3	71 ± 6	65 ± 18	67 ± 8	67 ± 6
[illegible]-old	0.02	24.5 ± 2.3	109 ± 7	57 ± 10*	52 ± 4	93 ± 4
	0.05	48.6 ± 5.2	121 ± 16	53 ± 14	58 ± 6	99 ± 14
	0.2	159.5 ± 20.2	109 ± 26	74 ± 27	58 ± 5	84 ± 8
	0.5	311.9 ± 43.4	107 ± 21	66 ± 14	62 ± 7*	84 ± 11
	2.0	1 188.8 ± 121.2	82 ± 16	43 ± 6	67 ± 4	69 ± 14

[illegible] slices [illegible] were preincubated for 30 min at 37°C under oxygen in Krebs-Ringer phosphate solution [illegible] 7.4) [illegible] with glucose (10 mmol/l) and with or without one of the metabolic inhibitors. [^{3}H] phenylalanine [illegible] varying amounts of unlabelled phenylalanine [illegible] were then added to give final concentrations of [illegible] mCi/l and 0.02–2.0 mmol/l respectively and the incubations were continued for 5 min. The results [illegible] means of 4–6 expts. with their standard deviations. The control [illegible] values are expressed as mmol/kg of [illegible] incubated tissue and the other values as percentages of the controls. Abbreviations: NaCN [illegible] sodium [illegible] cyanide, DNP [illegible] 2,4-dinitrophenol, NaIA = sodium iodoacetate.

[illegible] 0.01, [illegible] p [illegible] 0.05 when compared to corresponding controls in the two-tailed Student's t-test. [illegible] the age groups were compared, the control values differed significantly ($p < 0.01$) when the phenyl[illegible] concentrations were 0.02 and 0.05 mmol/l in the medium.

[illegible]

[illegible] amount of phenylalanine penetrated into the slices was calculated from the specific radioactivity of [illegible] phenylalanine in the medium and the radioactivity of the TCA supernatant. Because of the short [illegible] incubation period the incorporation of the label into proteins [illegible] negligible. According to thin-layer chro[illegible] matograms developed with phenol [illegible] water and n-butanol [illegible] acetic acid [illegible] water (3 : 1 : 4) less than 1 [illegible] of it was [illegible] metabolically degraded. In addition, the high medium-to-tissue ratio (50 : 1) ensured that the radioactivity [illegible] the incubation medium did not decrease significantly during the incubation (Vahvelainen and Oja [illegible] 72). The results are expressed as mmol/kg of wet weight of incubated tissue or as percentages of the [illegible] control concentrations. The statistical significance of the differences was calculated [illegible] with the Student's [illegible] two-tailed t-test. The straight lines of regression of the phenylalanine influx on the ATP level were [illegible] estimated with the method of least squares. The slopes of the regression lines and the correlation [illegible] coefficients with their 95 [illegible] confidence limits and the analyses of the differences observed between them [illegible] were carried out with common statistical methods (Spiegel 1961).

Results

[illegible] Metabolic inhibitors and phenylalanine influx

[illegible] During the 5-min incubation in all concentrations of phenylalanine the brain cortex slices [illegible] of the 7-day-old rats took up slightly more phenylalanine from the medium than the slices [illegible] from the adult rats (Table I). The influx was increasingly saturated at high phenylalanine [illegible] concentrations. The initial influx into the slices from the adult rats appeared to be slightly [illegible] enhanced by 0.2 mmol/l NaCN at low phenylalanine concentrations, but it was diminished [illegible] by 30% at 2.0 mmol/l phenylalanine compared with the control (Table I). The influx was [illegible] by about half by 1.0 mmol/l NaCN and 0.1 mmol/l DNP. The inhibitors caused

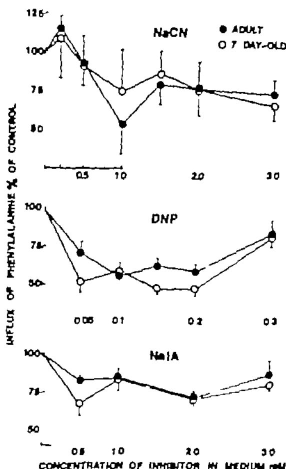


Fig. 1 Phenylalanine influx into brain slices of adult and 7-day-old rats in the of varying concentrations of metabolic inhib The slices were incubated as indicated in Ta but the concentration of phenylalanine was constant (0.2 mmol/l) in the medium. Th means of 4–6 expts., and the standard dev are expressed as the percentage of contro wet weight of incubated tissue was used reference

by NaIA (1 0 mmol/l) was stronger at the highest than at the lowest phenylalanine centration ($p < 0.05$) In the slices from the 7-day-old rats the effects of meta inhibitors were very similar to those in the slices from the adult rats.

When the phenylalanine concentration was kept constant (0.2 mmol/l) in the medium the concentrations of the inhibitors were varied, no striking differences between th groups were discernible (Fig. 1) The inhibition caused by NaCN reached its maxim 1 0 mmol/l NaCN DNP within the concentration range of 0.05–0.2 mmol/l inhibit influx by 30–45% and 40–55% in the slices from the adult and young rats, respectively influx was peculiarly greater in the presence of 0.3 mmol/l DNP than at lower concentrations. In the concentration range studied (0.5–3.0 mmol/l) NaIA inhibite influx by about 20–30%

Metabolic inhibitors and the levels of the studied compounds

The concentration of lactate in the control slices was about four times greater adult rats than in the 7-day-old ones (Table II) In the slices from the young ra concentration significantly ($p < 0.01$) increased in the presence of NaCN or DNP b in the slices from the adult rats. NaIA decreased the level of lactate more in the slices the adult rats than in those from the young ones.

II. Effect of metabolic inhibitors on the concentrations of lactate, ATP ADP AMP and creatine phosphate in brain cortex slices of adult and 7-day-old rats

f la	Metabolite	Control (mmol/kg)	NaCN 0.2 mmol/l ( )	NaCN 1.0 mmol/l ( )	DNP 0.1 mmol/l (⁻)	NaIA 1.0 mmol/l (..)
	Lactate	8.86 ± 1.29	97 ± 6	91 ± 10	102 ± 8	56 ± 2
	ATP	1.25 ± 0.25	80 ± 10	48 ± 5	98 ± 6	59 ± 6
	ADP	0.86 ± 0.16	86 ± 16	68 ± 16	74 ± 13	65 ± 3
	AMP	0.46 ± 0.08	58 ± 11	57 ± 9	44 ± 11	34 ± 4
	CP	0.99 ± 0.12	20 ± 17	0*	37 ± 10*	59 ± 10*
-old	Lactate	2.27 ± 0.78	140 ± 26	174 ± 34	235 ± 25	58 ± 43
	ATP	2.37 ± 0.44	91 ± 19	41 ± 7	37 ± 3	63 ± 2
	ADP	1.10 ± 0.17	59 ± 30	54 ± 5	53 ± 7	33 ± 7
	AMP	0.72 ± 0.04	60 ± 11	61 ± 14	42 ± 6	61 ± 10
	CP	1.20 ± 0.43	40 ± 5	4 ± 4	24 ± 7*	69 ± 14

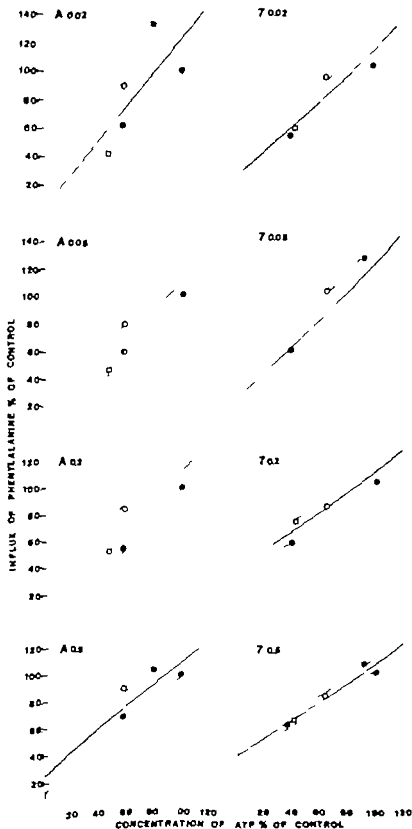
...lices were incubated for 35 min at 37°C under oxygen in Krebs-Ringer phosphate solution (pH 7.4) glucose (10 mmol/l) and with or without one of the metabolic inhibitors. The results are means of ...xpts. ...ith their standard deviations. The control values are expressed as mmol/kg of ...et weight of ...ssue and the other values as percentages of the controls. Abbreviations: NaCN sodium ...ide, DNP = 2,4-dinitrophenol, NaIA = sodium iodoacetate, CP = creatine phosphate
* $p < 0.01$ $p < 0.05$ when compared to corresponding controls in the two-tailed Student's t-test. When control values of the two age groups ...re compared, the values of lactate, ATP and AMP differed ...ficantly ($p < 0.01$).

...he concentrations of ATP and AMP in the control slices were higher in the young rats ...n in the adult rats, but the levels of ADP and cretaine phosphate did not differ signi...antly (Table II). All the metabolic inhibitors diminished the levels of these compounds ...larly in both the adult and young rats. The presence of 1.0 mmol/l NaCN 0.1 mmol/l ...NP or 1.0 mmol/l NaIA clearly diminished the levels of all the phosphate compounds. ...e concentration of 0.2 mmol/l NaCN was not very effective: only the decrements in ...TP and creatine phosphate were significant.

...orrelation between ATP level and phenylalanine influx

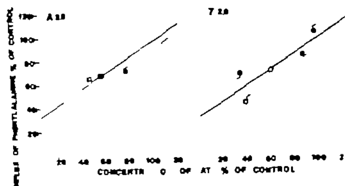
...linear correlation between the level of ATP and the influx of phenylalanine prevailed in ...e slices from both age groups (Fig. 2), the correlation coefficients differing from zero in ...e cases (Table III). The slopes of the regression lines of phenylalanine influx on ATP level ...ere systematically greater in the slices from adult rats at the lower phenylalanine con...ntrations, many differences between the age groups being statistically significant ...0.05). The slopes were also dependent on the concentration of phenylalanine in the ...edium. Generally at higher medium concentrations a certain decrement in the ATP level ...ciated with a relatively smaller diminution of the phenylalanine influx. The slope of the ...gression line at the lowest phenylalanine concentration differed significantly ($p < 0.05$) ...om the slope at the highest concentration in both age groups.

...There was no distinct correlation between the other phosphate compounds or lactate ...d the influx of phenylalanine.



A 0.02
7 0.02
A 0.05
7 0.05
A 0.2
7 0.2
A 0.5
7 0.5
INFLUX OF PHENYLALANINE % OF CONTROL
CONCENTRATION OF ATP % OF CONTROL

Fig. 2



2. Regression lines of phenylalanine influx and ATP level in brain cortex slices of adult and 7-day-old in the presence of metabolic inhibitors. The figures were drawn from the data in Table I and II. The ed lines represent the standard error of the estimates of phenylalanine influx on ATP level. The age of animals (A adult, 7 = 7-day-old) and the concentrations of phenylalanine (mmol/l) in the medium are ...ated in the upper lefthand corner of each individual graph. Symbols. ● = control exper., ■ = 0.2 mmol/l ...m cyanide, □ = 1.0 mmol/l sodium cyanide, ○ = 0.1 mmol/l dinitrophenol, ○ = 1.0 mmol/l sodium acetate in the medium.

Discussion

...culation of results

...re are several alternative ways to express results when tissue slices are studied. We ...ferred to use the final wet weight of incubated slices as a reference because it could ... more accurately assessed than any other possible reference. The share of [^{3}H] ...nylalanine which penetrated only into the extracellular spaces of the slices has not ... subtracted from the total influx since the size of the extracellular spaces cannot be ...sily measured with marker substances, in the presence of metabolic inhibitors in ...ticular. We have earlier determined the swelling and the inulin space of rat brain slices ...der the same type of incubation conditions (Lasko and Oja 1976). The metabolic ...bitors increased the swelling on most occasions, and the slices from adult rats always ...lled more. The inulin space was larger in the slices from 7-day-old rats under all ...ditions, but it proved to be a deceptive estimate for the extracellular space. When the ...ment results were also arbitrarily calculated per initial fresh weight of the slices and the ...asured inulin space was subtracted as a rough estimate for the extracellular space, the ...solute magnitudes of the results were altered of course, but our inferences were not ...luenced by this trial.

When we calculated the share of saturable intracellular uptake in the total influx at ...fferent phenylalanine concentrations by using the transport constants estimated earlier by ... (K_t 0.66 and 0.22 mmol/l, V_{max} 101 and 44 mmol/kg/min for adult and 7-day-old rats, ...spectively) (Vahvelainen and Oja 1977), it appeared to be ...r about four-fifths at the ...nylalanine concentration of 0.02 mmol/l and only one-third at 2.0 mmol/l. At the lowest ...mino acid concentrations the effects of metabolic inhibitors mainly reflect changes in ...e saturable intracellular transport, and at the highest concentrations it predominantly ...ows the nonsaturable penetration into the slices.

TABLE III Correlation coefficients between the concentrations of ATP and phenylalanine and the (regression coefficients) of the least square regression lines of phenylalanine influx and concentration.

Age of animals	Conc. of phenylalanine in medium (mmol/l)	Correlation coefficient	Regression coefficient
Adult	0.02	0.72 (0.46–0.87)	1.22±0.50
	0.05	0.87 (0.73–0.94)	1.05±0.25
	0.2	0.79 (0.58–0.90)	1.04±0.34
	0.5	0.82 (0.63–0.92)	0.86±0.26
	2.0	0.90 (0.79–0.96)	0.64±0.13
7-day-old	0.02	0.93 (0.84–0.97)	0.84±0.13
	0.05	0.88 (0.74–0.95)	0.91±0.21
	0.2	0.94 (0.86–0.97)	0.67±0.11
	0.5	0.97 (0.93–0.99)	0.68±0.07
	2.0	0.87 (0.72–0.94)	0.64±0.16

The coefficients with their 95% confidence limits were calculated from the data presented in Table I as described in "Material and Methods" The regression lines are presented in Fig. 4.
Different from the corresponding value of the other age group ($p < 0.05$).

Effects of metabolic inhibitors

Cyanide restrains cell respiration by inhibiting cytochrome oxidase and therefore prob a shift from aerobic to anaerobic metabolism (Keilin 1928 Stotz *et al* 1938). It drastically affects the structures of nerve cells (Ibata *et al* 1971) and may thus alter permeability of nerve cell membranes. A slightly enhanced influx of phenylalanine at mmol/l cyanide in the presence of diminished levels of creatine phosphate and ATP indicate such permeability changes. Inhibition of the influx at 2.0 mmol/l phenylal probably results from diminution of the extracellular space (Laakso and Oja 1976) high NaCN concentration (1 0 mmol/l) reduced the influx at all phenylalanine concen tions and decreased both the intracellular transport and the extracellular space.

The main metabolic effect of DNP is the uncoupling of oxidative phosphoryl (Loomis and Lipmann 1948), but it may also stimulate a latent ATPase activity in mitochondria (Lardy and Wellman 1953) DNP also changes the conformation of membrane proteins of mitochondria and alters the permeability of mitochondrial m branes (Carafoli and Rossi 1967 Weinbach and Garbus 1968) It is not known wh such conformational changes occur in neural plasma membranes. An unexpected s increase in the phenylalanine influx at a DNP concentration of 0.3 mmol/l (see Fig. 1) c reflect an increased penetration of phenylalanine down to its concentration gradient a damaged cell membranes. This explanation is in accordance with the diminished inhib provoked by DNP as the phenylalanine concentration increased (Table I)

Iodoacetate is a classical inhibitor of glycolysis at the level of glycerose 3-phos (Cori *et al.* 1948) The production of lactate is inhibited *in vitro* already at low concentra (0.01 mmol/l) of iodoacetate (McMurray et al. 1957), whereas higher concentrations exert other diverse effects on cell metabolism. The uptake of the

ls, proteins, DNA and RNA may all be inhibited by iodoacetate (Heald 1953, Glazer Weber 1971 a). According to Guroff *et al.* (1961) iodoacetate does not alter the cell mbrane permeability for passive diffusion, neither are the swelling or the size of the n space of brain cortex slices altered by iodoacetate (Laakso and Oja 1976). However our present study the NaIA-induced inhibition of phenylalanine influx was significantly ater at the higher than at the lower phenylalanine concentration in both age groups. metabolic effects of iodoacetate may be augmented by high concentrations of phenyl-nine because phenylalanine also inhibits glycolysis in brain slices (Woodman and Dwan 1961 Glazer and Weber 1971 b).

Phenylalanine influx was less affected by 1.0 mmol/l NaIA than by 1.0 mmol/l NaCN 0.1 mmol/l DNP. Also Banay-Schwartz *et al.* (1971) have reported that the NaIA uced decrease in the level of ATP in brain slices is generally more pronounced than the comitant decrease in the influx of amino acids.

relation between ATP level and phenylalanine influx

e correlation between the ATP level and the phenylalanine influx was more linear than ected since the inhibitors also produce other metabolic effects in addition to affecting ATP concentration. The positive correlation prevails also at high phenylalanine centrations and this phenomenon suggests that ATP may play a role in the factors ch influence the passive fluxes of amino acids in brain slices and the tissue swelling ring incubation. Because the swelling of the brain slices during incubation parallels the rease of intracellular sodium (Piccoli *et al.* 1971), the role of ATP in the distribution of ali metal ions in the slices may manifest itself in this finding. The intracellular swelling y increase when the level of ATP decreases, and the enlargement of intracellular lume may displace some extracellular fluid. So the amount of the amino acid diffunded to the extracellular space diminishes and correlates to the ATP content of the slices.

From the above discussion one can conclude that the positive correlation between ATP el and phenylalanine efflux also at low amino acid concentrations may be a secondary enomenon and do not prove that ATP were a direct source of energy for the active ansport of amino acids.

The regression lines of phenylalanine influx on the ATP level of the slices were systemati-lly steeper for the adult than for the young rats at the low phenylalanine concentrations. he following explanations are possible: (1) The extracellular space in the slices from young ats is larger than that in the slices from adult rats (Laakso and Oja 1976), and the diffusion f phenylalanine into extracellular spaces should be independent of the ATP level. This ifference in extracellular spaces between the age groups may not be large enough to onstitute the only explanation however. (2) Passive energy-independent diffusion across ell membranes could be faster in the slices from 7-day-old rats. We have determined earlier owever that the transmembrane diffusion constants for phenylalanine are equal in lices from 7-day-old and adult rats (Vahvelainen and Oja 1972). (3) In the control incuba-tions the content of ATP was higher in the slices obtained from 7-day-old rats. After a similar percentage reduction in ATP there is still more ATP left in the slices of 7-day-old rats. This extra ATP is not, however, able to support adequately the phenylalanine

influx The breakdown rate of ATP in the brain tissue of young rats is slower than brain tissue of adult rats (Takagaki 1974) If ATP really in some way furnishes energy amino acid transport the rate of utilization of ATP in the slices of immature brain is a limiting factor for phenylalanine influx, but not the level of ATP as such.

Conclusions

The developmental changes in the influx of amino acids into cerebral cortex slices seem not to be connected to the developmental changes in energy metabolism. metabolic inhibitors which disturbed different steps in energy formation had very effects on phenylalanine influx in the two age groups. The observed positive correlation between the ATP level in the slices and the influx of phenylalanine did not prove ATP was a source of energy for amino acid transport. In any case, the level of ATP did limit the influx of phenylalanine into the slices from immature brain More probable for the changes of amino acid transport in brain slices during development may be changes in alkali metal concentrations and distributions in the slices (Piccoli *et al.* Franck and Schoffeniels 1972), because as in other tissue preparations, the transport amino acids in brain slices is at least partly dependent on optimal alkali metal concentrations (Margolis and Lajtha 1968). Furthermore, the changes in the properties of carrier systems of cell membranes are possible during the maturation of brain tissue

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Hepatic triacylglycerol and fatty acid biosynthesis during hypoxia *in vivo*

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Abstract

KINNULA, V L., M J SAVOLAINEN and I HASSINEN *Hepatic triacylglycerol and fatty acid biosynthesis during hypoxia in vivo* Acta physiol scand. 1978 *104* 148–155

Hepatic fatty acid biosynthesis and the activity of phosphatidate phosphohydrolase, the rate-limiting enzyme of triacylglycerol biosynthesis, were studied after hypoxic periods of 1 and 7 days under hypobaric conditions at 40.8 kPa. Phosphatidate phosphohydrolase activity increased 2-fold in the soluble fraction of the liver after one day at 40.8 kPa but had returned to normal by 7 days. This was accompanied by a significant increase in liver triacylglycerol and *sn*-glycerol-3-phosphate. The phosphatidate phosphohydrolase activity increased continuously over 7 days in the pair-fed controls, probably due to the restriction on food. Measured as *in vivo* incorporation of ^3H_2O into lipids, the hepatic fatty acid synthesis increased somewhat in acute hypoxia, but returned to normal values during 7 days of hypoxia. Plasma free fatty acids increased markedly after 24 h in the fasting controls (90 %) with a smaller increase in the hypoxic group (50 %) due to peripheral lipolysis. Hepatic glycogen stores decreased in the hypoxic and fasting animals both after 1 and 7 days. It is concluded that hypoxia induces the accumulation of fat in the liver at least partly as a consequence of an increase in phosphatidate phosphohydrolase activity in the soluble fraction of the liver

Key words: Liver hypoxia, triacylglycerols, phosphatidate phosphohydrolase, fatty-acid synthesis

In a previous paper (Kinnula and Hassinen 1978), we observed that severe hypoxia (40.8 kPa) causes triacylglycerol accumulation in the liver of the rat. Similarly ischemia or anoxia can lead to a lipid accumulation in the heart and aorta (Bryant *et al* 1958, Scheuer and Brachfeld 1966, Briggs and Glenn 1976), and hypoxia-induced fatty liver in suckling rats has been observed (Chiodi and Bass 1969) One reason for this hypoxia-induced steatosis seems to be a reduction in fatty acid oxidation in the liver (Kinnula and Hassinen 1977), while other possible reasons might be lipolysis from the adipose tissue or increased lipogenesis in the liver The rate of lipogenesis *in vivo* can be reliably studied by measuring the incorporation of tritium from ^3H_2O into hepatic lipids (Lowenstein *et al.* 1975). results also indicate that under certain conditions hepatic triacylglycerol accumulation is preceded by an increase in the activity of phosphatidate phosphohydrolase (E.C. 3 1 3.4) and it seems that this enzyme constitutes the rate limiting step

-erol and triacylglycerol by liver cells (Lamb and Fallon 1974 a, b, Savolainen 1977). -refore further studies concerning hepatic triacylglycerol accumulation in hypoxia were ducted by determining the rate of fatty acid synthesis *in vivo* and the activity of phospha-ite phosphohydrolase in hypoxic rat liver.

Material and methods

-gents. Standard reagents were obtained from E. Merck AG, Darmstadt, Germany. Coenzymes, glycerol--sphate dehydrogenase and lactate dehydrogenase were purchased from Boehringer GmbH, Mann-a, Germany and ^3H_2O from NEN Chemicals GmbH, Dreieichenhain, Germany. Phosphatidic acid obtained from Koch-Light Laboratories, Colnbrook, Bucks, U.K.

-imals and hypoxic experiments. Adult Sprague-Dawley rats from the Department own colony were . Both sexes were used, and the age of the animals varied between 3–4 months. Their food consisted standard rodent chow (Astra-Ewos Ab, Sodertälje, Sweden). 3 groups of the animals were studied in case, forming *ad libitum*-fed control, pair-fed control and hypoxic groups. For the lactate and -erol-3-phosphate determinations the rats were anaesthetized with pentobarbital (60 mg/kg, intraperit-eally). After cannulation of the trachea the respiration was controlled with rodent respiration pump -rvard Apparatus Company Model 680). The respiratory rate was fixed at 40 cycles per min, and the -l volume calculated from the equation log $= -1.51 + 0.735$ log m, where m is the body weight of the -mal in grammes and the total volume in millilitres (Kleinman and Radford 1971). The animals breathed -n air for 30 min in the control experiments and 8% O_2 in nitrogen in the hypoxic experiments. In order -btain metabolite concentration figures from partially adapted animals, the animals in one group of -eriments were kept in hypobaric pressure chamber at 40.8 kPa for 1 or 7 days before the 30-min -iod in the respirator under 8 O_2. *sn*-Glycerol-3-phosphate and lactate concentrations in the liver -ples were measured after the 30 min period in the respirator. In studying fatty acid biosynthesis or -sphatidate phosphohydrolase activity hypoxia was induced in the hypobaric pressure chamber.

Phosphatidate phosphohydrolase. After a period of one or seven days in the hypobaric pressure chamber 40.8 kPa the rats were decapitated and their livers removed immediately and placed in ice-cold 0.225 M -rose containing 1 mM EDTA and 50 mM Tris-HCl, pH 7.5. The liver was homogenized in 4 volumes the same medium with glass homogenizer fitted with teflon pestle. The homogenate was centrifuged 15 000 *g* for 20 min, after which the supernatant was centrifuged at 105 000 *g* for 60 min. After $(NH_4)_2SO_4$ -atment (0.23 g/ml, 0°C) and further centrifugation at 10 000 *g* for 20 min, the surface of the pellet was -dly rinsed with 1 ml of medium containing 0.25 M sucrose, 0.5 mM dithiothreitol and 20 mM Tris-HCl, 7.4 and then dissolved in this buffer. The phosphatidate phosphohydrolase activity of the $(NH_4)_2SO_4$ -ction was determined by the method of Hosaka *et al.* (1975) as modified by Savolainen (1977). The -ount of inorganic phosphate released was determined by the method of Ames and Dubin (1960). The -zyme activity was expressed as nmoles inorganic phosphate liberated per min and g of wet weight.

Fatty acid synthesis in vivo. The rate of hepatic lipogenesis *in vivo* was measured by determining the -corporation of tritium from ^3H_2O into long-chain fatty acids as described by Lowenstein *et al.* (1975). -one series of experiments the animals were kept in the hypobaric pressure chamber at 40.8 kPa for seven -ys or pair-fed for the same period. At 9.00–9.30 a.m. on the eighth day they were anaesthetized with -her and injected intracardially with 5 mCi of ^3H_2O in 0.9 NaCl. They were then returned to the hypo--aric pressure chamber still at 40.8 kPa, and liver and blood samples were obtained exactly 60 min after -jection under ether anesthesia. In another series of experiments two groups of normal animals were -jected with ^3H_2O, one group was kept at normal atmospheric pressure, and the other group exposed to -e pressure of 40.8 kPa. About 0.5 g of liver in liquid nitrogen was weighed rapidly and homogenized in 19 -lumes chloroform–methanol (2:1 by volume) using an Ultra-Turrax homogenizer. Fatty acids and non--aponifiable lipids were extracted and counted for radioactivity in toluene-based scintillating solution -Lowenstein *et al.* 1975). The specific radioactivity of the body water of each animal was determined by -measuring the tritium radioactivity of sample of blood, and performing the calculations described by -Jungermann and Sparth (1969).

Metabolite concentrations. At the end of the 30-min period of controlled ventilation in the respirator -under approximately the same partial pressure of oxygen as in the hypobaric pressure chamber, one lobe of the liver was freeze-clamped by the technique of Wollenberger *et al.* (1960). After extraction of the -vine with 6 perchloric acid the supernatant was neutralized to pH 6.0 with 3.75 M K_2CO_3 con-

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Hepatic fatty acid biosynthesis and the activity of phosphatidate phosphohydrolase, the rate-li[mit]ing enzyme of triacylglycerol biosynthesis, were studied after hypoxic periods of 1 and 7 days under hypo-baric conditions at 40.8 kPa. Phosphatidate phosphohydrolase activity increased 2-fold in the soluble fraction of the liver after one day at 40.8 kPa, but had returned to normal by 7 days. This was accompanied by a significant increase in liver triacylglycerol and sn-glycerol-3-phosphate. The phosphatidate phospho-hydrolase activity increased continuously over 7 days in the pair-fed controls, probably due to the restriction on food. Measured as *in vivo* incorporation of $^{3}H_{2}O$ into lipids, the hepatic fatty acid synthesis increased somewhat in acute hypoxia, but returned to normal values during 7 days of hypoxia. Plasma free fatty acids increased markedly after 24 h in the fasting controls (90 %) with a smaller increase in the hypoxic group (30 %) due to peripheral lipolysis. Hepatic glycogen stores decreased in the hypoxic and fasting animals both after 1 and 7 days. It is concluded that hypoxia induces the accumulation of lipids in the liver at least partly as a consequence of an increase in phosphatidate phosphohydrolase activity in the soluble fraction of the liver.

Key words: Liver, hypoxia, triacylglycerols, phosphatidate phosphohydrolase, fatty-acid synthesis

In a previous paper (Kinnula and Hassinen 1978), we observed that severe hypoxia (40.8 kPa) causes triacylglycerol accumulation in the liver of the rat. Similarly ischemia or an... can lead to a lipid accumulation in the heart and aorta (Bryant *et al.* 1958, Scheuer and Brachfeld 1966, Briggs and Glenn 1976) and hypoxia induced fatty liver in suckling... has been observed (Chiodi and Bass 1969). One reason for this hypoxia-induced ste... seems to be a reduction in fatty acid oxidation in the liver (Kinnula and Hassinen 1... while other possible reasons might be lipolysis from the adipose tissue or increased ...genesis in the liver. The rate of lipogenesis *in vivo* can be reliably studied by measuring incorporation of tritium from $^{3}H_{2}O$ into hepatic lipids (Lowenstein *et al.* 1975). Pre... results also indicate that under certain conditions hepatic triacylglycerol accumul... is preceded by an increase in the activity of phosphatidate phosphohydrolase (E.C. 3.1.-... and it seems that this enzyme constitutes the rate-limiting step

1 Effect of hypoxia (40 8 kPa) on hepatic lipogenesis in vivo. For measurement of the rate of lipogenesis the rats were injected intracardially with 1.8 mCl (acute expts.) or 5 mCl (chronic expts.) of ^3H_2O and killed 60 min later during which time the animals referred to as hypoxic were kept at 40 8 kPa. Animals which had been at 40 8 kPa for 7 days or pair-fed or *ad libitum* control animals at normal atmospheric pressure were referred to as chronic expts. The radioactivity was determined from isolated lipid fractions (Lowenstein *et al.* 1975). Values are means ± S.D. from 4 separate animals.

	Acute experiments		Chronic experiments		
	Control	Hypoxia	*Ad lib.* control	Pair-fed control	Hypoxic
y acids (nmol of $_2$O incorporated/sec/g wt. of liver	47 4±14.7	76 2±31.7	55.9±28.9	38 4±16 1	43.7±5.5
...ions of non-...onifiable lipids ...mol of ^3H_2O incorpo-...ed/sec/g wet wt. of liver	30 0±11.0	25.3±9 1	27.8±7.9	37 7±20.7	35.5±16.7

...oxia, but the triacylglycerol concentration had not increased any further after 48 h ...‡ µmol/g wet wt. of liver). The difference in triacylglycerol concentration between the ...-fed and hypoxic animals was significant (p < 0.01) after 7 days, but not after one day *Fatty acid synthesis.* Acute hypoxia increased the hepatic lipogenesis, but this was not ...tistically significant (Table I). Seven day's hypoxia had not effects on the rate of fatty ...d synthesis when compared with the normal or pair-fed controls (Table I).

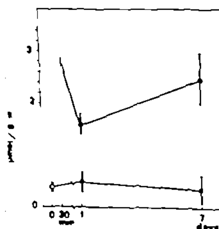


Fig. 2

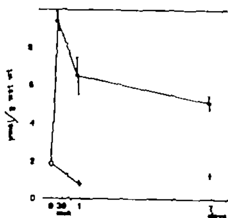


Fig. 3

Fig. 2. Effect of hypoxia on hepatic sn-glycerol-3-phosphate concentrations. For the 30-min period the animals were in respirator under 8% O_2 and for the 1-day and 7-day expts. they were exposed to pressure of 40 8 kPa for the given length of time prior to the 30-min period in the respirator under 8 O_2. Values are means ± S.E. from 4–5 separate expts. Symbols as in Fig. 1

Fig. 3. Effect of hypoxia on hepatic lactate concentrations. Values are means ± S.E. from 4–5 separate Experimental conditions and symbols as in Fig. 1 and 2.

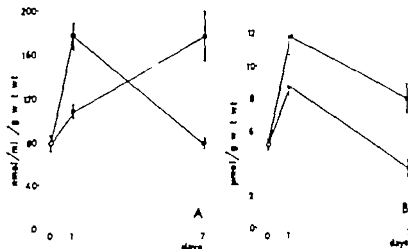


Fig. 1 Effect of hypoxia (40.8 kPa) on hepatic phosphatidate phosphohydrolase activity (A) and tri-glycerol concentration (B). Values are means ± S.E. from 7–8 separate expts. Symbols: O control; hypoxic and ■—■ pair fed.

taining 0.5 M triethanolaminehydrochloride. Lactate and sn-glycerol-3-phosphate concentrations determined in the neutralized perchloric acid extract as described by Gawehn and Bergmeyer (1974) Hohorst (1970). In determining the hypoxic values of the following metabolites only the hypobaric pressure chamber was used. Liver glycogen was determined from the freeze-clamped tissue according to van Handel (1965) and triacylglycerols according to Carlson (1963). Plasma free fatty acids were measured by the method of Duncombe (1963) and blood glucose by that of Bergmeyer *et al.* (1970).

Statistical methods. The two tailed student's t-test for independent means was used for the statistical analysis, P values of < 0.05 being considered to indicate statistical significance.

Results

Weight response. Because of the reduction in food consumption in the hypobaric pressure chamber pair-feeding of the animals was used. On their first day in the chamber 60 the rats did not eat at all and the average food consumption of the rest was only 3.5 g no pellets were given to the pair-fed animals during the first 24 h (= 24-h fasting gr The weight loss during seven days was 15.4% in the rats kept at 40.8 kPa and 14.6 in pair fed controls. Two out of the 95 rats used died in the hypobaric pressure chamber.

Phosphatidate phosphohydrolase. The activity of this enzyme increased significantly d 24 h in hypoxia, from a control value of 78.3 nmol/min/g wet wt of liver to 177 nmol/ wet wt. ($p < 0.001$). Fasting for 24 h similarly resulted in a significant increase, to 109 min/g wet wt. ($p < 0.02$) but the difference between the fasting and hypoxic groups nevertheless highly significant ($p < 0.001$) (Fig. 1). After 7 days in severe hypoxia n ferences in the activity of this enzyme could be detected any longer between the co (*ad libitum*), and hypoxic groups, but a higher value was noted in the pair-fed grou comparison with the *ad lib* controls ($p < 0.05$) (Fig. 1).

Triacylglycerol concentration. The increase in the concentration of liver triacylgly can be observed in Fig. 1. An increase in phosphatidate phosphohydrolase activity (p < and liver triacylglycerol concentration ($p < 0.05$) could be observe the f

ary factors (Hill *et al.* 1958), and therefore the nutritional status of the animals must arefully controlled in order to reveal the specific effects of other factors.

The hepatic activity of phosphatidate phosphohydrolase, a rate-limiting enzyme in ylglycerol and triacylglycerol biosynthesis in the liver (Lamb and Fallon 1974 a, b), eases after acute ethanol administration (Savolainen 1977), after subtotal hepatectomy in sham-operated rats (Mangiapane *et al.* 1973). There are no previous reports on its vity during hypoxia.

The present results demonstrate that hepatic phosphatidate phosphohydrolase activity eases in hypoxia, and that there is also an increase in the triacylglycerol concentration. increase in phosphatidate phosphohydrolase activity was concomitant with an increase he concentration of sn-glycerol-3-phosphate. It has been previously shown that hepatic ylglycerol accumulation correlates with phosphatidate phosphohydrolase activity (Savolen 1977), and also with hepatic sn-glycerol-3-phosphate activity (Savolainen and Hasn 1977), and the present results suggest that the same relations exist in hypoxia, although time-course of the changes was not studied in detail. The semi-starvation of the pair normoxic control animals resulted in a slow steady increase in the activity of phosphate phosphohydrolase, but only a transitory initial increase in the triacylglycerol concention. Moreover an increase in phosphatidate phosphohydrolase activity has been obred previously during starvation (Vavrečka *et al.* 1969), under conditions in which there o increase in hepatic triacylglycerol formation (Vavrečka *et al.* 1969 Park *et al.* 1972). explanation is at present available for the behaviour of phosphatidate phosphohydrolase ring starvation.

The possibility that peripheral lipolysis may induce an accumulation of triacylglycerols the liver in hypoxia cannot be excluded, for catecholamine liberation also increases in oxia (Cannon and Hoskins 1911 Maher *et al.* 1975). In the present study a significant rease in the plasma free fatty acid concentration was observed in acute hypoxia after h, but the concentration was significantly higher still in the 24-h fasting animals. This obably means that fatty acid liberation from the adipose tissue in hypoxia is at least partly e to the state of semistarvation. The smaller increase in the plasma free fatty acid concention in the hypoxic animals may indicate that hypoxia itself has some inhibitory effect lipolysis, an effect that has been observed in perfused rat hearts at least (Crass and per 1975). I the present case the increased lipolysis was not reflected in the hepatic acylglycerol concentration, which did not differ significantly from the controls in the 24-h sting of 7-day pair-fed groups.

The rate of hepatic fatty acid synthesis increased somewhat in acute hypoxia, but returned normal alues during one week of hypoxia. Under the latter conditions, however the patic triacylglycerol concentration was more than twice that in the pair-fed controls. On e basis of the present results it is not possible to exclude the possible contribution of an creased fatty acid synthesis rate in the triacylglycerol accumulation, but it seems that ring the prolonged hypoxia of seven days the triacylglycerol accumulation is not regulated the rate of fatty acid synthesis. A positive correlation has previously been bserved between hepatic lipogenesis and glycogen concentration (Salmon *et al.* 1974), but this could t be erified in the present work on hypoxia. Lactate has also been implicated in lipo-

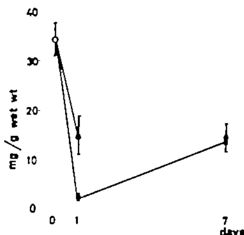


Fig. 4. Effect of hypoxia on hepatic glycogen concentrations. Values are means ± S.E. from 4-5 separate ... Experimental conditions and symbols as in Fig 1

Other metabolites The hepatic *sn*-glycerol 3-phosphate concentration increased significantly in acute hypoxia ($p < 0.001$) from a control value of 0.367 μmol/g wet wt of l... to 2.84 μmol/g after 30 min under 8 % O_2. The concentration was smaller ($p < 0.1$) at ... than after 30 min in hypoxia but was still significantly elevated ($p < 0.001$) compared ... the controls. After seven days the concentration was 2.46 μmol/g, a value higher than t... of the controls ($p < 0.001$) and also higher than the value after 24 hours ($p < 0.01$) (Fig ... Liver lactate concentration increased in acute hypoxia after 30 min, from a control value ... 1 85 μmol/g wet wt of liver to 9 42 μmol/g ($p < 0.001$) After 7 days hypoxia the concentration was 5 11 μmol/g, higher than the control value ($p < 0.001$) but lower than the concentration in the non-adapted animals after 30 min of hypoxia ($p < 0.02$) (Fig. 3) The liver glycogen concentration decreased significantly during the first 24 h in hypoxia ($p < 0.01$), and ... still low after seven days in hypoxia ($p < 0.01$) (Fig. 4). Still lower glycogen concentrations ($p < 0.001$) were observed in the 24-h fasting control group (Fig. 4) Blood glucose did not change in hypoxia after either 24 h or 7 days when compared with the *ad libitum* control... the values being 7 00 ± 0 08 mmol/l (mean ± S D n = 4) in the control group, 9 40 ... mmol/l (mean ± S D n = 4) after 24 hours at 40 8 kPa and 8 41 ± 1.22 mmol/l (mean ± S D n = 4) after seven days at 40.8 kPa. The plasma free fatty acid concentration was significantly ($p < 0.05$) higher after 24 h in hypoxia *i.e* 0.54 ± 0 10 mmol/l (mean ± S.D., n = 4) as compared with the *ad libitum* controls (0.36 ± 0 16 mmol/l mean ± S D., n = 8), but ... after 7 days. The plasma free fatty acid concentration was similarly significantly higher ... the fasting animals (0 70 ± 0 13 mmol/l, mean ± S D n = 8) after 24 h than in the fed controls ($p < 0.01$) and the hypoxic group ($p < 0.05$)

Discussion

The present results confirm the recently reported increase in hepatic triacylglycerol ...tration in hypoxic hypoxia (Kinnula and Hassinen 1978), although the increase was ... smaller possibly due to the different rat strain used. Strain differences are also indicated ... the lower mortality of the Sprague-Dawley rats in hypoxia. Lipid synthesis is affected ...

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genesis and it has been suggested that it may have a regulatory role not connected with precursor nature (Salmon *et al.* 1974). No correlation was found between the lactate concentration and the rate of lipogenesis in the present study.

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acetylcholine (ACh) (Heiwall *et al.* 1975) and choline acetyl transferase (CAT) ...dewska *et al.* 1975) in the sciatic nerve was blocked by COL or VIN. Both these drugs ...ct with cytoplasmic microtubules and bind to the tubule protein *in vitro*. (*e.g.* Borisy ...aylor 1967 Malawista, Sato and Bensch 1968). Since the effect of mitotic inhibitors on ...was paralleled in some neuron systems by a decrease in the number of axonal micro-...s (Banks *et al.* 1971, Fink *et al.* 1973), the anti-transport effect was considered to ...e to a specific destruction of the microtubules.

...ever both COL and VIN have biochemical effect on cytoplasmic organelles and ...es other than microtubules and their subunit, tubulin (*e.g.* Feit and Barondes 1970, ...n *et al.* 1970). Therefore the effects of COL and VIN on AXT may be due to other ...s in the cell and not directly related to their binding to microtubules.

...test whether inhibition of AXT by mitotic inhibitors is related to their effect on micro-...es, isomers to mitotic inhibitors, having a documented less effect on microtubules, have ...tested. Thus, the isomer to COL, lumiCOL has a much less binding to microtubules ... COL (Bryan 1972) and has also a much less effect on the AXT of labelled proteins ...son and McClure 1974, 1975). Another pair of substances is podofyllotoxin (POD) ...ts isomer picropodofyllin (picPOD). POD is an efficient inhibitor of AXT and binds to ...lin like COL (Wilson 1970, Paulson and McClure 1975a, Banks and Till 1975). The ...er has less binding to tubulin than POD and also a much less antitransport effect ...lson and McClure 1975 b).

...ese two pairs of substances, COL and lumiCOL on the one hand, and POD and picPOD ...he other hand, have now been used to study the involvement of axonal microtubules in ...reviously demonstrated anterograde AXT of ACh, AChE and CAT in rat motor nerves. ...reliminary report of these studies has been published (Dahlström *et al.* 1975).

Materials and methods

...nals. For the COL-lumiCOL experiments male rats of the hooded strain (250–300 g) were used. Male ...gue-Dawley rats (250–300 g) were used in the other experiments.

...perative procedure. Under ether anesthesia the sciatic nerves bilaterally ...ere exposed about 10 mm ...w the *for. suprapiriformis*. Under the control of ... dissection microscope the test substances were in-...ed subepineurally with ... 32 gauge needle. The volume of each injection was 3–5 μl ... [illegible] spread 3–5 mm ...ch direction. COL or POD and their respective isomers (lumiCOL and picPOD) were tested in pairs, ...hat the mother substance ...as injected into one nerv... and the isomer into the contralateral nerve (after ...ng tubes) of the same animal, in equimolar concentrations. 2–3 h after the injection, the animals were re-...sthetized and ... crush operation (*a.m.* Lubińska 1959) was performed 15 mm distal to the injection level ...Fig. 1). After 12–13 h the rats were made unconscious by ... blow on the head (for ACh expts.), or de-...itated (for enzyme assays), the nerves ...ere dissected out and chilled on an ice-cooled glass-plate prior to ...ing.

...hemicals. COL (Sigma) was used to prepare lumiCOL according to Wilson and Friedkin (1966). ...1 M solution of COL in 95 ... ethanol ...as irradiated for 36 h ...ith 366 nm UV-light from ... HPW 125 W



...1. Schematic illustration of ...ection level, crush site 15 mm ...ally and nerve segments used ... ACh (A–C) and CAT or ...Pasesth. (5-c).

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Further evidence for the involvement of microtubules in the proximo-distal intra axonal transport of acetylcholine and related enzymes in rat sciatic nerve

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Abstract

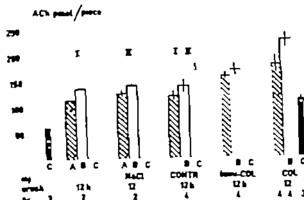
HEIWALL, P.-O., P. A. LARSSON and A. DAHLSTRÖM. *Further evidence for the involvement of microtubules in the proximodistal intra-axonal transport of acetylcholine and related enzymes in rat sciatic nerves.* Acta physiol. scand. 1978. *104.* 156–166.

The two mitotic inhibitors colchicine (COL) and podofyllotoxin (POD) and their respective isomers lumiCOL and picropodofyllin (picPOD) were tested for their effect on the intraaxonal transport (AXT) of acetylcholine (ACh) and the cholinergic enzymes in rat sciatic nerve. The mitotic inhibitors and their isomers were dissolved in saline + 10 % ethanol (COL and lumiCOL) or dimethylformamid (DMFA) (POD and picPOD) and injected (3–5 μl) subepineurally into the sciatic nerve. As controls the vehicle (saline + 10 % ethanol or DMFA) alone were injected into some rats. 2 h later a crush operation was performed distal to the site of injection. The accumulation of ACh or the two enzymes, ACh-esterase (AChE) and cholineacetyltransferase (CAT), in the nerve segment proximal to the crush (12 h before death) was used as a measure of the AXT. COL and POD were very effective in inhibiting AXT of all 3 substances, while the isomers, lumiCOL and picPOD, were essentially without effect on AXT in equimolar concentrations. The effects on AXT of the 4 test substances thus appear related to their affinity to bind to tubulin, which is several orders of magnitude higher for COL and POD than for their isomers. The results further support the view that intact microtubules are essential for AXT of both membrane-bound (AChE) and soluble (CAT) enzymes, as well as of ACh in rat motor nerves.

Key words: Intra-axonal transport, cholinergic enzymes, acetylcholine, mitotic inhibitors, isomers, tubulin affinity

The intra-axonal transport (AXT) of proteins, organelles, enzymes and certain transmitters has for many years been suggested to depend on intact microtubules in the axon (for ref. see Dahlström 1971, McClure 1972, Heslop 1975, Hanson and Edström 1978). This theory was supported by the early experiments with local application of colchicine (COL) or vinblastine (VIN) on adrenergic nerves and sciatic nerve to study the AXT of noradrenaline (NA) granules and acetylcholinesterase (AChE) (Dahlström 1968, Kreutzberg 1969, Banks et al. 1971). The proximo-distal AXT of both these substances was blocked by COL or VIN. Later it was shown that also the anterograde transport of isotope-labelled proteins (e.g. J

The ACh content of 12 h d rat sciatic nerve after the neural injection, 2 h before of saline 10 ethanol), lumi-COL (0.1 M) or COL M) Various segments as ted in Fig. 1. Individual or mean ± S.E. are given. ferent from control A-seg- at p 0.05 and -nt from lumi-COL B and C ms at p 0.01 and p 0.005 ively.



l (group I, Fig. 3). The A and B segments in this group contained 116–137 pmol/10 mm h is a normal amount for 10 mm segments at that level (see also Fig. 6). In nerves in- d with the vehicle for the test substances, (physiological saline with 10% of ethanol) and ned 12 h before death (group II, Fig. 2), the ACh-accumulation in segment C was lar to that in group I. No change in ACh-content of segments A and B was caused by vehicle administration (compare groups I and II, Fig. 2). Since the 2 groups were very lar in ACh content, they were taken together as a control group (I + II in Fig. 2). The ction of 0.1 M lumiCOL caused a small increase in both segments A and B (significant for segment A), but the ACh-accumulation in segment C was similar to that in the trol group. The injection of 0.1 M COL, on the other hand, caused a marked increase in h content in segments A and B, and a significant decrease in the ACh-accumulation in ment C 12 h after crushing (to 110 ± 6 pmol, p 0.005 compared to lumiCOL).

AChE. The results in Fig. 4 are expressed in per cent of the enzyme activities in segments 2 d 3 of the saline-treated group in each separate expt. a) because this level of the nerve is branched (see Fig. 1), and b) because the AChE-accumulation does not reach segment 2 ring 13 h after crushing. The results in Fig. 4 are based on 4 expts., and the 100%-value ried between 3 457–8.640 pmol of ACh split/min/5 mm nerve in the various expts. When en together 100 ± 9% corresponded to 6.234 ± 578 pmol of ACh split/min/5 mm (n = 18). he injection of the vehicle between the segments 3 and 4 (see Fig. 1) did not induce any noticeable change in AChE-activity in the segments 3–5 as compared to a normal uncrushed atic nerve. In the C-segment, the enzyme activity was increased to 212 ± 18% at 13 h after ushing. In the lumi-COL-treated group, the enzyme activity in segment C was 166 ± 20%. us, it appeared somewhat lower than in the saline group but the difference was non- gnificant. Following COL-treatment, the AChE activities of segments 4 and 5 were clearly creased as compared to the two other groups, and the activity in segment C was only 0 17.5. Thus, no accumulation of enzyme activity appeared to have occurred during the h following crushing (significantly different from the C-segment in the lumiCOL-group at 0.025).

CAT. The results in Fig. 5 are based on 3 different expts. and expressed in per cent of the activity in segments 2 and 3 of the control (saline) group for reasons indicated above

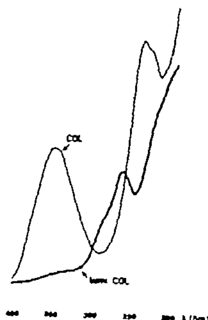


Fig. 2. The UV absorbance spectra between 200-400 nm of COL and lumi-COL after irradiation at 366 nm.

mercury lamp (Philips). To control that no COL was left in the solution the ultraviolet absorbance was read between 200–400 nm on a Zeiss spectrophotometer. In parallel a COL solution, treated but not irradiated, was also read. Only samples in which the typical absorbance peak of COL at 355 nm disappeared were used (Fig. 2). The ethanol present in the solutions was evaporated, and the lumi-COL weighed and kept in the cold until use. 0.1 M solution of the two substances were prepared just before injection in saline with 5–10% ethanol. In one group of control animals (Fig. 3–5) the vehicle alone was injected.

POD (Aldich Chemical Comp. Inc.) was used to prepare the picPOD according to Wilson and Friedman (1967). A solution of 200 mg of POD in 10 ml of methanol and 1 ml of a 78% NH was stirred for ... and cooled to yield a flocculent precipitate (picPOD). This was washed twice with 10 ml portions of absolute ethanol and dried *in vacuo*. The determination of the melting point was used to control the purity of the picPOD (mp. 221–224°C) prepared from POD (mp. 112–114°C). Prior to injection the picPOD was dissolved in dimethyl formamid (DMFA, Merck) to a final concentration of 0.1 M. In groups of animals the vehicle alone was injected and its effect compared to that of saline (Fig. 6–8).

Assay methods: *ACh* was assayed on 10 or 5 mm segments of nerve as indicated in Fig. 1. 2–4 pieces were pooled for each assay. Following extraction according to MacIntosh and Perry (1950) the samples were assayed on a guinea pig ileum preparation (Blaber and Cuthbert 1961). An antihistamine (Mepyramine) was added to the Krebs solution used in the gut bath. Schild's (1949) four point assay was used to calculate the ACh-activity of the samples. ACh was pharmacologically identified by inactivation with purified AChE (Worthington Biochemical Corp., New Jersey) and by atropine inhibition of the responses of the gut to standard and sample. The activity measured was thus "ACh-like" but will be referred to as ACh in the following.

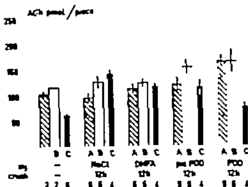
AChE and *CAT* were assayed in 5 mm segments (Fig. 1) according to the method of Tuček (1970). Both enzymes could be measured in the same nerve segment.

For statistical analysis of the results Student's t test was used.

Results

COL-lumiCOL experiments

ACh. The ACh-content of a normal uncrushed 5 mm C-segment was 61 ± 1 pmol (Fig. 3). In nerves which were only crushed for 12 h, the content of the C-segment was as ...

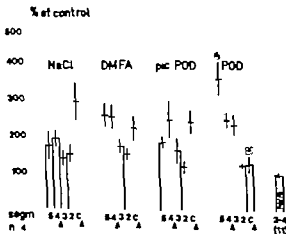


The ACh content of normal, intact and crushed rat sciatic nerve after the sub-[illegible] injection (3–5 μl) of saline (NaCl), (DMFA), picPOD (0.1 M) or POD [illegible]). Various segments as indicated in Mean ± S.E. are given. *indicate dif- from picPOD group at $p < 0.025$.

picPOD experiments

In normal, uncrushed sciatic nerve (see Fig. 6), the 5 mm C-segment contained 63 ± 3 [illegible] of ACh (5). The 10 mm A and B segment contained 104 ± 3 and 117 ± 21 pmol, [resp]ectively. In order to compare the effect of solvent used for the test substances (DMFA) the mechanical effect of the injection trauma 2 control groups with 12 h crushes were

AChE



▲ site of inj 2h before crush

▲ site of crush 13h before death

[Fi]g. 7. The AChE-activity in rat sciatic nerve after the subepineurial injection (3–5 μl) of saline (NaCl), [ve]hicle (DMFA), picPOD (0.1 M) or POD (0.1 M). Arrows indicate sites of injection and crush. Mean ± S.E. given. 100 [illegible] 3.236 ± 239 pmol of ACh split/min/5 mm nerve.
[*] indicate different from NaCl at p [illegible] 0.025–0.05
[illegible] different from DMFA at p [illegible] 0.025
[illegible] indicate different from picPOD at p [illegible] 0.01 and p [illegible] 0.025 respectively

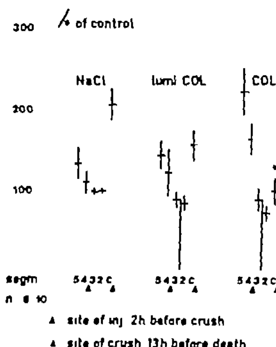


Fig. 4 The AChE-activity in rat sciatic after the subepineural injection (3-5 μl saline + 10 ethanol (NaCl), lumi-COL COL (0.1 M). Arrows indicate site of injection and crush. Mean ± S.E. are given = 6 234 ± 578 pmol of ACh split/mm/5 nerve. Indicates difference between COL and COL groups at p < 0.025.

(AChE-section). 100 ± 2 % corresponded to 1 140 ± 27 pmol of ACh formed/min/5 mm ment. In the C-segment of the control group, 110 ± 5 % of CAT-activity was present at after crushing while in the lumiCOL group the level was 115 + 3 % in the same segment the COL treated group, however the C-segment contained only 99 ± 4 % (p < 0.005, compared to the lumiCOL group) while segments 4 and 5 contained somewhat higher CAT activities than the corresponding segments of the 2 other groups (Fig. 5)

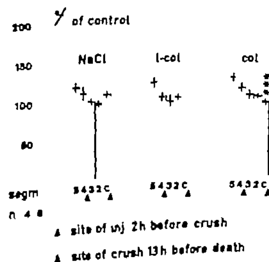
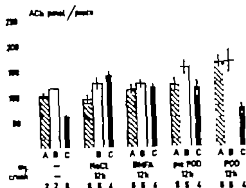


Fig. 5 The CAT-activity in rat sciatic after the subepineural injection of 3-5 μl saline + 10 ethanol (NaCl), lumiCOL M) or COL (0.1 M). Arrows indicate site of injection and crush. Mean ± S.E. are given 100 % = 1 140 ± 27 pmol of ACh formed mm nerve. Indicates different from C-segment of lumi-COL group at p 0.0



6 The ACh content of normal, intact and crushed rat sciatic nerve after the subneural injection (3–5 µl) of saline (NaCl), -le (DMFA), picPOD (0.1 M) or POD M). Various segments as indicated in 1. Mean ± S.E. are given. *indicate dif at from picPOD group at p 0.025

D-picPOD experiments

In normal, uncrushed sciatic nerve (see Fig. 6), the 5 mm C-segment contained 63 ± 3 pmol of ACh (n 5). The 10 mm A and B segment contained 104 ± 3 and 117 ± 21 pmol, respectively. In order to compare the effect of solvent used for the test substances (DMFA) the mechanical effect of the injection trauma 2 control groups with 12 h crushes were

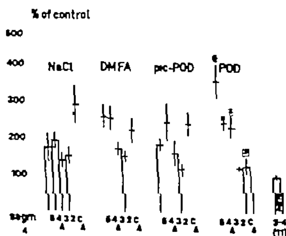


Fig. 7 The AChE-activity in rat sciatic nerve after the subepineural injection (3–5 µl) of saline (NaCl), hyde (DMFA), picPOD (0.1 M) or POD (0.1 M). Arrows indicate sites of injection and crush. Mean ± S.E. given. 100 1,236 ± 259 pmol of ACh split/min/5 mm nerve.
) indicate different from NaCl at p 0.025–0.05
‡ indicate different from DMFA at p 0.025
indicate different from picPOD at p 0.01 and p 0.025 respectively

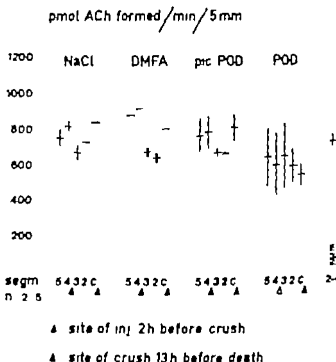


Fig. 8 The CAT-activity in rat sciatic nerve after the subepineural injection (3–5 μl) of saline (NaCl), vehicle (DMFA), picPOD (0.1 M) or POD (0.1 M). Arrows indicate sites of injection and crush. Individual values or mean ± S.E. are given. Normal 5 mm nerve contains 724–34 pmol of ACh formed/min/5 mm nerve. ... indicates different from C segment of the picPOD group at p < 0.025

included one group with DMFA injections in the B-segment and one with saline injections (see Fig 6). In the saline injected group 143 ± 8 pmol was present in segment C while the segment of the DMFA injected group contained 115 ± 13 pmol of ACh. Thus, a small but insignificant decrease in ACh-accumulation had probably occurred after DMFA-treatment. When the test substances were injected into the nerve the C-segment contained 115 ± 13 pmol of ACh in the picPOD-group while in the POD-group only 75 ± 9 pmol was present (difference between the two test substances at p 0.025). The A and B segments of the POD group contained somewhat higher ACh-content than the same segments of the other groups (difference of the A-segment against the picPOD-group p 0.025)

AChE. The results shown in Fig. 7 represent 2 expts. and are expressed in per cent of non-crushed uninjected segments (2–5) for each experimental occasion. 100 ± 5.4% corresponds to 3436 ± 259 pmol of ACh split/min/5 mm segment. As in the ACh part of this expt. (see above), two control groups were included one injected with saline and the other with DMFA. The DMFA injection appeared to cause a) an increase in segments 4 and 5 (above the site of injection) as compared to the saline group and b) a decrease in the amount of enzyme activity which had accumulated in segment C during the 12 h of crushing. The differences were not significant however. In the group injected with picPOD the AChE-activities of the various segments was about the same as in the DMFA-treated group. The POD-group ho

-T showed significant differences as compared to the other 3 groups. The AChE present in segment C was only 127±22% as compared to 240±28 and 223±31% in the picPOD and DMFA-group, respectively ($p<0.025$). In segments 3–5 there was an increased AChE activity particularly in segment 5 (356±43%) as compared to the picPOD-group or the DMFA-group (p 0.01–0.025).

CAT. The results in Fig. 8 represent 2 experiments and are expressed in pmol of ACh formed/min/5 mm nerve. A normal 5 mm segment contained 724±34 pmol of ACh formed/min/5 mm segment ($n=5$). The control groups, injected with saline or DMFA, showed rather similar enzyme activities. The CAT-activity of the C-segments at 12 h after crushing was thus as a mean 842 and 808 pmol of ACh formed/min/5 mm in the 2 groups, respectively (Fig. 8). In the picPOD-treated nerves the CAT-activity of segment C was 811±75%, and the enzyme activities of the other segments was similar to those in the 2 other groups. After POD-treatment, however, the CAT-activity of the C-segment was only 540±61 pmol of ACh formed/min/5 mm (different from the picPOD-group at $p<0.025$). In the other segments, the CAT-activities showed a large variation (Fig. 8).

Discussion

The biological method to assay ACh has been discussed earlier (Dahlström *et al.* 1974). The assay performed, i.e. incubation with purified AChE and atropine block of the gut, strongly suggest that the measured activity was ACh. This is also supported by results from chemical determination of ACh in rat sciatic nerve by Ulln *et al.* (1976) which are in excellent agreement with our values.

The radiometrical methods for assaying AChE and CAT in the same homogenate have been discussed by Tuček (1974). The enzyme activities observed in this study agree well with those previously observed by other investigators, except that the AChE activities in some groups have been unusually high (up to about 100 µmol of ACh split/h/g of nerve, as compared to 56 µmol per h/g of rat sciatic nerve as reported by Kasá *et al.* (1973)). The CAT activity varied in this study between 8.5–13 µmol of ACh formed/h/g, while previous investigators found in rat sciatic nerve a CAT-activity corresponding to 8–10 µmol of ACh formed/h/g (Fonnum 1969, Saunders *et al.* 1973).

As seen in Fig. 3–5 the injection of 0.1 M COL into the sciatic nerves 2 h before the crush operation caused a decreased accumulation of ACh, AChE and CAT in segment C, just above the crush. For all 3 substances this decrease, when compared to the lumiCOL group, was significant. Since the amount of ACh, AChE and CAT appeared increased around and above the areas of injection of COL, but not (or to a much less extent) of lumiCOL, the results suggest that COL, but not lumiCOL, had interrupted the proximo-distal AXT of ACh and the two enzymes, thus reducing the accumulations further distally in the C-segment. LumiCOL, a structural isomer of COL, has many biological properties in common with COL, but differs markedly in its affinity to bind to the microtubule subunit protein, tubulin. LumiCOL has less than 1/100 of the affinity of COL to tubulin (Wilson and Friedkin 1967, Wilson *et al.* 1974, *cf.* also Paulson and McClure 1975). The transport-inhibiting capacity of these two agents thus appear to be correlated to their affinity to tubulin. The fact that

lumiCOL appeared to have a small effect on the accumulation of AChE-activity (Fig 4 in agreement with the fact that this drug does have a small affinity for tubulin.

The other pair of substances tested, POD and picPOD gave results essentially simil those discussed above. The vehicle itself DMFA, appeared to interfere to some degree the transport of especially ACh and AChE, as judged from the small decrease in accumul amounts in the C-segments (Fig. 6 and 7). However the addition of picPOD (0.1 M) to DMFA did not cause any further disturbances in the transport of ACh and AChE, w POD-injection had very marked effects on both ACh and the enzymes. Above and at the areas of injection of POD both ACh and AChE-activity increased, and in the C-segm the accumulation of all 3 substances was significantly lower than in the picPOD group. Th picPOD had much less effect on the axonal transport of both ACh and the enzymes t had POD in equimolar concentrations. POD binds 135 times more to tubulin than picPOD (Wilson and Friedkin 1967 Paulson and McClure 1975). Thus, also in this c there seems to be a correlation between the tubulin affinity of the substances and its tr port inhibitory effect

The above substances were injected in seemingly very high concentrations, if compar with the mM-concentrations used in *in vitro* expts. (Edström and Mattson 1972, Paulson McClure 1975 Hansson and Edström 1977) However the few µl of test solution injec into the nerve spread over about 10 mm (*cf* Dziegielewska *et al* 1975) and probably eq librated in a volume of at least 10 µl Therefore, the tissue concentration of these substanc probably was in the order of 25 mM which is less than an order of magnitude higher th the concentration of *e g* COL used *in vitro* to block axonal transport in frog sciatic nerv (5 mM Edström and Hansson 1974)

The results of the present study show that lumiCOL and picPOD had much less inhibit effect on rapid AXT of AChE and ACh than COL and POD and are in agreement with res by other investigators Thus, Paulson and McClure (1974, 1975) could arrest rapid AXT labelled proteins *in vitro* by COL, POD and VIN but had no effect of lumiCOL, or picPO In rabbit optic nerves protein transport was inhibited by COL but not by lumiCOL (Fi 1974) The transport of noradrenaline storage vesicles in cat hypogastric nerves was block *in vitro* by COL but not by lumiCOL (Banks and Till 1975). These results strongly sugg that microtubules are involved in rapid AXT of various substances, but do not rule out possibility that both COL and POD may interfere with AXT by acting on processes n directly related to microtubules.

We have previously reported that both COL and VIN interfere with the transport ACh (Heiwall *et al* 1975) and CAT (Dziegielewska *et al* 1975) in rat sciatic nerve, suggesti the participation of microtubules in transport of both these substances. This suggestion h been further strengthened in the present study The interesting thing is that also the transp of the soluble enzyme CAT appears to be dependent on microtubules (see Fig. 5 and 8) rat sciatic nerve this enzyme appears to move with the so called slow phase of AXT bu very small fraction may theoretically move faster (*cf* Saunders *et al* 1973). The dependen on intact microtubules also of this probably slow transport (*i.e* a slow rate of mean transp velocity) is in agreement with the "microstream hypothesis" of Gross (1975) or the "transp filament theory" of Ochs (1971).

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Suppression of autonomic postganglionic discharges by pentobarbital in dogs, with or without endotoxemia

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Abstract

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al effects of pentobarbital (8 mg/kg) on autonomic efferent and afferent discharge rates were studied 16 dogs under morphine-chloralose anesthesia. Half of the dogs were given endotoxin *E. coli* (1 mg/kg) ore pentobarbital. The postganglionic cervical vagal efferentation of all the dogs decreased as did the ganglionic cardiac sympathetic efferentation. The heart rate of the dogs given endotoxin decreased, ile an increase in heart rate with abolition of respiratory arrhythmia, was observed in dogs without lotoxin. The aortic pressure of the former dogs dropped while it fell only slightly in the latter ones. aortic arch baroreceptor activity decreased while the changes of left atrial B-type receptor activity re not significant. The changes of the left atrial and central venous pressures were slight but those of the lmonary arterial pressure generally paralleled the changes in the aortic pressure. Pentobarbital, ac rdingly seems to exert both sympatholytic and vagolytic effects. These explain the heart rate changes, well as the impaired cardiac contractility it evokes. The obvious impairment of cardiovascular control chanisms by pentobarbital should be seriously considered in investigations into the cardiovascular conl

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entobarbital has been, and still is, one of the most widely used anesthetics in cardiovascular xperimentation. However the effects of the drug are generally disregarded in the inter retation of the data obtained. This is due to the fact that the circulatory and the autoomic effects of pentobarbital are not completely known (Manders and Vatner 1976).

Lacking direct evidence, it has been difficult to differentiate the direct effects of pentoarbital on the heart and blood vessels, from those on the autonomic nervous system which, gain, may secondarily modulate cardiovascular functions (Horwitz 1977). Thus, the oncept of the vagolytic effect of the drug (Morrison, Walker and Richardson 1960, Vidrio and Pardo 1967 Page and Hoff 1969) emanates from the varying degrees of tachycardia it vokes in experimental animals (Van Citters, Franklin and Rushmer 1964 Gilmore 1965). But under special circumstances, the drug reportedly elicits even bradycardia (Swanson and Shonle 1930). On the other hand, a sympathetic activation by pentobarbital has been suggested from observations on its hypertensive effects (Rushmer Van Citters and Franklin

1963 Priano Traber and Wilson 1969 Sawyer Lumb and Stone 1971 Golding et al In dissonance with this are findings of decreased vascular smooth muscle (Harvey 1975), of decreased myocardial contractility and of impaired left ventricular function following injections of pentobarbital (Vatner and Braunwald 1975). The cardiac depression in response to barbiturates is greatly enhanced in states of circulatory shock (Fi 1943 Becher 1951 Van Bergen 1965 Enderby 1965 and Wolfson 1966). Apart cardiac actions (Cox 1972 a), a pentobarbital-induced sympatholysis may be impl in effecting this myocardial depression.

In order to gain insight into the conflicting data just outlined, we have studied the barbital-induced changes in the spontaneous discharge rates of autonomic nerve f controlling the heart and circulation in dogs under morphine-chloralose anesthesia. The responses of the heart rate and blood pressures were also recorded To upset the c status and reflex functions, half of the dogs were given endotoxin prior to adm of the barbiturate. Thus experiments were performed on two groups of dogs, a co group being in as normal condition as the method warrants and an endotoxic group in a state of shock induced within a few minutes.

Material and methods

The experiments were carried out on 26 mongrel dogs, weighing from 7 to 29 kg. Following premedication with subcutaneous morphine hydrochloride (1 mg/kg) the dogs were anesthetized with intrav α-chloralose (100 mg/kg) dissolved in warm physiological saline. After the induction of anesthesia animals were intubated, and the tracheal tubing was connected to a volume controlled respirator thoracic aorta and the inferior caval vein were cannulated via the femoral vessels. Arterial blood sa were taken anaerobically duri g the operative phase for acid-base and blood gas determinations. possible shifts in these were corrected to physiological limits, by adjusting the pulmonary ventilation by i.v administration of sodium bicarbonate solution This was considered mandatory because the responsiveness of the cardiovascular system to catecholamines is depressed by acidosis (Darby et al and because changes in the arterial carbon dioxide tension modify the sympathetic tone (Millar and 1965).

A left thoracotomy was performed and the upper lobe of the left lung was resected. The pulmonary ar and left atrium were cannulated via the vessels of the resected lobe All pressures were recorded strain gauge transducers (Hewlett Packard 267BC) and amplified with carrier amplifier (Hewlett Pa 8805B).

In the thorax, a mineral oil pool was created from the parietal pleura. The sympathetic postgangl discharges were recorded from the f bers of the third cardiac nerve On the neck the left vagus nerve exposed and cervical efferent vagal impulses with cardiac rhythm were recorded. The aortic arch b receptor and the left trial B-type receptor fiber activity were also recorded on the neck

One half of the dogs (13) were given intravenous endotoxin *Escherichia coli* 1 mg/kg 5–15 min before recordings were started.

Pentobarbital (Nembutal® Abbott) was given to the dogs given endotoxin (endotoxic dogs) and the dogs without the toxin (control dogs). The dose levels were 7–9 mg/kg (mean 8.1 ± 1 1 S.D.), and drug was injected within 15 s. All the neural and circulatory variables were recorded on magnetic tape an instrumentation tape recorder (PI 6200, Precision Instrument, U.S.A.). The recordings were st 1 2 min before the pentobarbital injection and continued for p to 3 min 20 s. Thereafter recordings lasting 20 s, were started at 5 9 and 15 min.

The action potentials recorded were calculated with a multichannel nalyser (Nokia LP 4840, N Electronics, Finland). The means of relative changes (per cent of control levels) in all recordings of same fiber type were calculated for each 20 s period. The t-test was used to test the significance of changes and of the differences between the two groups of dogs.

The methods used are detailed elsewhere (Halinen 1976).

I. Mean control values of heart rate and blood pressures. The means of control period heart rates (HR), systolic (AP syst) and diastolic aortic pressures (AP diast), and systolic (PAP syst) and diastolic pulmonary arterial pressures (PAP diast) in dogs under morphine-chloralose anaesthesia (control dogs) and in dogs under morphine-chloralose anaesthesia given endotoxin (endotoxic dogs). Statistical significance of differences between the two groups of dogs is given in the table.

	HR beats/min	APsyst mmHg	APdiast mmHg	PAPsyst mmHg	PAPdiast mmHg
rol	115	142	102	25.1	15 0
	± 52	± 14	± 11	± 4.1	± 3.7
	0 001	0.001	0 001	0.20	0 01
otoxic	202	80	62	31 1	24 7
	± 43	± 21	± 20	± 6.7	± 7.0

Results

general, the control heart rates and pulmonary arterial pressures in the endotoxic dogs eeded those in the control dogs, while the reverse held true for the aortic pressures ble I).

The endotoxic dogs were acidotic (pH_a 7.265 ± 0 081 mean ± S.D) as compared with trol dogs (pH_a 7.373 ± 0.042 p 0.001). The plasma bicarbonate of the former dogs was 3 ± 2 8 mmol/l and that of the latter ones 20.8 ± 2.5 mmol/l, ($p < 0.001$). The mean arterial od oxygen tension of the endotoxic dogs (80 ± 23 mmHg) was lower ($p < 0.001$) than that control dogs (132 ± 32 mmHg). The mean arterial carbon dioxide tension of both groups s about 30 mmHg.

The responses to pentobarbital in a typical dog are illustrated in Fig. 1 There was aortic essure oscillation and respiratory arrhythmia, which faded off soon after pentobarbital jection. Along with its stabilization, the heart rate mounted to a higher level. Concur ntly the vagal cardiac efferentation showed a more marked suppression than did the mpathetic one. The average baroreceptor activity scarcely changed, but following pento- rbital, as the aortic pressure and heart rate were stabilized, the fluctuation of the baro- ceptor impulse activity diminished

Control dogs. Fig. a summarizes the changes in the cardiovascular variables and nerve scharge rates following the pentobarbital injection. The mean heart rate (13 dogs) increased om 115/min to 149/min (p 0 05), 1 min after the pentobarbital injection. Following sub- quent cardiac deceleration, equally increased heart rates were seen at 9 and 15 min. Con- rrently the mean aortic pressure (13 dogs) fell from 142/102 mmHg to 127/94 mmHg p 0 05). The pulmonary arterial pressures showed a slight initial increase ($p < 0.05$) from 5/15 mmHg but then showed trend to decrease.

The cervical vagal efferentation (8 dogs; Fig. 2 a and 3) was decreased 30 s after pento- arbital injection It decreased to a minimum of 60 per cent of the control level ($p < 0.01$) at 3 min after the injection. Later on, the vagal efferentation recovered towards the control level (Fig 2).

1963, Priano, Traber and Wilson 1969, Sawyer, Lumb and Stone 1971, Golding *et al.* ... In dissonance with this are findings of decreased vascular smooth muscle responses (Harvey 1975), of decreased myocardial contractility and of impaired left ventricular function following injections of pentobarbital (Vatner and Braunwald 1975). The cardiac depression in response to barbiturates is greatly enhanced in states of circulatory shock (E. 1943, Becher 1951, Van Bergen 1965, Enderby 1965 and Wolfson 1966). Apart from cardiac actions (Cox 1972 a), a pentobarbital-induced sympatholysis may be ... in effecting this myocardial depression.

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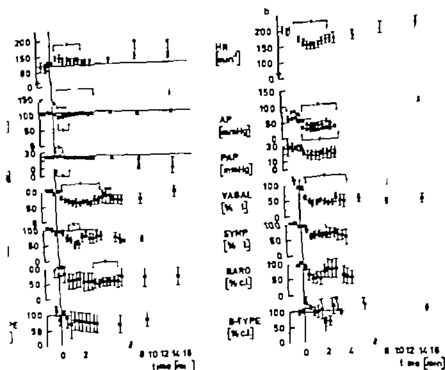


Fig. 2 a. Effects of pentobarbital (8 mg/kg) given at zero time on dogs under morphine-chloralose anesthesia (control group). HR = heart rate; AP = systolic and diastolic aortic pressures; PAP = systolic and diastolic pulmonary arterial pressures; VAGAL = cervical vagal efferent discharges; BARO = aortic arch baroreceptor discharges; B-type = left atrial B-type receptor discharges; % c.l. = mean impulse frequencies as per cent of control level frequency. The vertical bars indicate ±1 S.E. and the horizontal lines the control of each parameter. Statistical significance (p < 0.05) is labeled with asterisks.

Fig. 2 b. Effects of pentobarbital (8 mg/kg) injected at zero time on dogs under morphine-chloralose anesthesia and endotoxemia (endotoxin E. coli 1 mg/kg; endotoxin group). For abbreviations see Fig. 2 a.

0.001) and it was attained at 1–3 min. The vagal discharges showed no recovery in the follow-up period.

The cardiac sympathetic efferentation (3 dogs, Fig. 2 b) decreased during the cardiac acceleration to a minimum of 55 per cent of the control level (p < 0.05), at about 2 min.

The aortic baroreceptor activity (5 dogs, Fig. 2 b) decreased with the falling aortic pressure (p < 0.05). The nadir of 53 per cent occurred at 2 min.

The mean left atrial B-type receptor discharge rate (7 dogs, Fig. 2 b) showed considerable fluctuation following pentobarbital injection. The minimum discharge rates were encountered at 2 min, but at the end of the experimental period their firing was at the control level.

Discussion

Lacking direct instrumentation for recordings of autonomic discharges in conscious animals, general anesthesia was used in the present expts. The premedication dose of morphine, even if small, may augment the vagal tone (Page and Hoff 1969). Chloralose was selected for

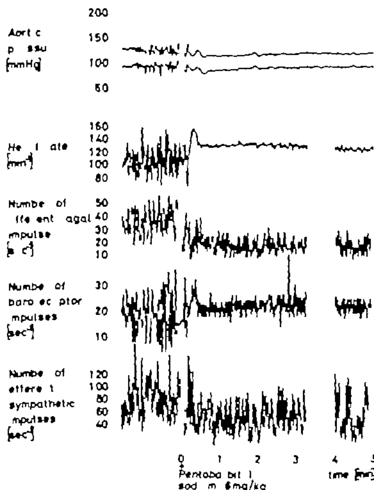


Fig. 1 Circulatory and nervous responses of a dog ... morphine-chloralose anesthesia ... pentobarbital injection (6 mg/...

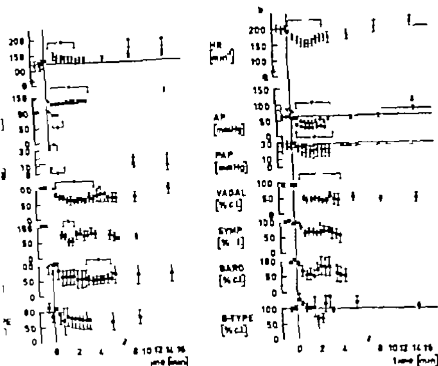
The cardiac sympathetic efferentation (5 dogs Fig. 2 a and 4) decreased significantly ($p < 0.05$) 1–2 min after the pentobarbital injection. During its maximum reduction, the average discharge rate was 52 per cent of the control level (Fig. 2 a).

The aortic baroreceptor activity (5 dogs 2 a) decreased concomitantly with the ... aortic pressure. At 4 min, the baroreceptor activity was 51 per cent of the control level ($p < 0.05$).

The mean left atrial B-type receptor discharge rate (3 dogs 2 a) showed a trend to decrease (N.S.) after the pentobarbital injection.

Endotoxin dogs The heart rate (13 dogs Fig. 2 b) decreased from 202/min to 151/... ($p < 0.001$) following pentobarbital. The lowest values occurred at 1–2 min. At 15 min the heart rate had accelerated to 217/min. The mean aortic pressure (13 dogs) initially dropped from 80/62 mmHg to 43/30 mmHg in this group of dogs. The decrease was highly significant ($p < 0.001$) Subsequently the mean aortic pressure mounted to above the control level. The mean pulmonary arterial pressure decreased from 31/27 mmHg to 25/19 mmHg (N.S.) after the pentobarbital injection (4 dogs Fig. 2 b).

The cervical vagal efferentation (8 dogs Fig. 2 b) began to decrease 30 s after the pentobarbital injection. The minimum average discharge rate was 41 per cent of the control



a. Effects of pentobarbital (8 mg/kg) given at zero time on dogs under morphine-chloralose anesthesia (control group). HR heart rate; AP systolic and diastolic aortic pressures, PAP systolic and diastolic pulmonary arterial pressures, VAGAL cervical vagal efferent discharges; BARO aortic arch baroreceptor discharges, B-type left atrial B-type receptor discharges, c.l. mean impulse frequencies as per cent of control level frequency. The vertical bars indicate ± 1 S.E. and the horizontal lines the control of each parameter. Statistical significance (p 0.05) is labeled with asterisk.

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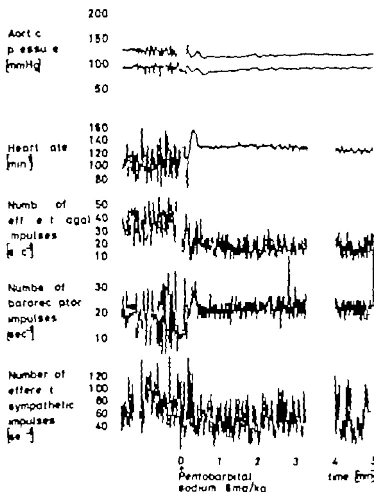


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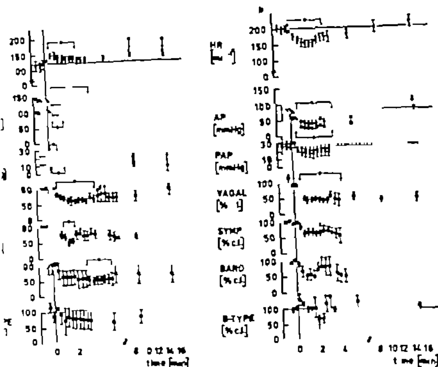
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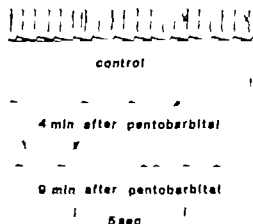
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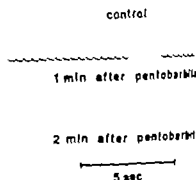


Fig. 3

Fig. 4

Fig. 3. Action potentials recorded from an efferent cervical vagal nerve fiber and the aortic pressu before and after pentobarbital injection in a dog under morphine-chloralose anesthesia (control g The horizontal lines indicate the aortic pressure levels of 200, 100 and 0 mmHg.

Fig. 4. Discharges in an efferent cardiac sympathetic fiber and the aortic pressure curve before an pentobarbital injection in a dog under morphine-chloralose anesthesia and endotoxemia (endotoxi 1 mg/kg). The horizontal lines indicate the aortic pressure levels of 100 and 0 mmHg.

basic anesthesia because it leaves the autonomic balance and reflexes largely intact (1960 Greisheimer 1965 Cox 1972 b). Admittedly the possibility exists that the obs responses reflect an interaction of pentobarbital and morphine-chloralose anesthesia. H ever the heart rate and blood pressure responses to pentobarbital (tachycardia and hypotension) were similar to those it usually elicits in dogs without underlying morph chloralose anesthesia (Cox 1972 a). With these statements in mind we believe our re warrant the following facets of evaluation.

Control state. The slight tachycardia in the control group of dogs was probably a r of an activation of the sympathetic nervous system, by the surgical trauma inherent in preparative procedures. The endotoxic dogs were in a state of shock as judged from basic heart rate, arterial pressure and acid base balance (Ettinger and Suter 1970, Lef Linden and Norman 1971).

Pentobarbital effects. The small pentobarbital dose (8 mg/kg) injected, immediately pressed both the vagal and the sympathetic efferentation. The mean heart rate in the co dogs increased while a decrease in heart rate occurred in the endotoxic dogs. Like the l rate changes in these two groups, the arterial pressure changes differed as well. In control dogs a slight, though significant, fall was observed, whereas a marked drop oc in the endotoxic dogs. The pulmonary arterial pressure of the former group slightly rose, fell in the latter group. The aortic arch baroreceptor activity decreased equally in b groups. Concomitantly the sympathetic efferentation was reduced and the respirat arrhythmia was abolished. This set of events suggests depression of the baroreceptor ref No significant changes were found in the atrial B-type receptor discharges and the left at mean pressure.

The observed decrease of the vagal efferentation accounts for the tachycardi finding with pentobarbital anesthesia. This substantiates the earlier indi

vagolytic action of the drug (cf. Introduction). The existing evidence regarding the [sympath]etic responses to pentobarbital is also indirect but conflicting. The suppression [of] cardiac sympathetic tone observed following pentobarbital in the present experiments [may] be more apparent than real. It possibly resulted from a reduction of antecedent sym[path]etic activation by anesthesia and surgical trauma. Yet, this sympathetic suppression [explains] the bradycardic reactions occasionally observed in response to pentobarbital [(Johnson] and Shoale 1930, Cox 1972 a). It also explains the impairment of cardiac con[trac]tility and left ventricular functions by pentobarbital (Cox 1972 a, Vatner and Braunwald [197]5, Manders and Vatner 1976). The myocardial depression may be reinforced by direct [effe]cts of pentobarbital on the heart. It is interesting to note, however that i.v. thiopental [sodi]um affects heart rate, arterial pressure, and cardiac contractility like pentobarbital, but [thio]pental given directly into the left circumflex coronary artery has no demonstrable [card]iac depressant action (Horwitz 1977). In addition, the possible sympathetic suppression [con]nected with the induction of anesthesia, could be responsible for the greatly enhanced [circ]ulatory depressant effects of barbiturates in oligaemia (Halford 1943, Becher 1951 [an]d Bergen 1965, Wollson 1966) and for the precipitation of cardiac arrest by these agents [(Ab]derby 1965). The sympatholytic origin of this phenomenon, however remains obscure [as o]utlined above.

[T]he present results are compatible with the concept on the dominant role of the vagal [ne]rve in controlling the heart rate in normal circumstances, while in the presence of heart [fail]ure the adrenergic component is more operative (Braunwald 1974). Also in endotoxin [sho]ck, the sympathetic tone excites the heart more than the increased vagal tone suppresses [(H]Halmen 1976). However the methods used do not warrant quantifying the relationship [bet]ween changes in efferent autonomic activity and changes in heart rate.

[C]oncluding remarks. Subanesthetic doses of pentobarbital, accordingly have both sym[pat]holytic and vagolytic effects. These explain the heart rate changes by the drug and the [dec]rease in cardiac contractility as well. The baroreceptor reflexes, too, are apparently [de]pressed by pentobarbital. Thus, pentobarbital seems to impair the cardiovascular control [m]echanisms. This explains the susceptibility to barbiturates of patients in circulatory shock. [Ob]viously the results from experimentation on circulatory physiology in animals with [b]arbiturate anesthesia must be interpreted with extreme care. The same holds for extrapola[tio]ns of such experimental data to clinical situation.

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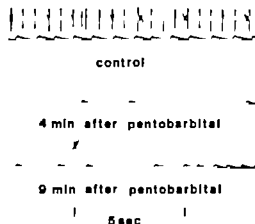


Fig. 3

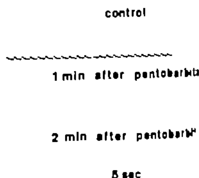


Fig. 4

Fig. 3 Action potentials recorded from an efferent cervical vagal nerve fiber and the aortic pres before and after pentobarbital injection in dog under morphine-chloralose anesthesia (control g The horizontal lines indicate the aortic pressure levels of 200, 100 and 0 mmHg.

Fig. 4 Discharges in efferent cardiac sympathetic f ber and the aortic pressure curve before and pentobarbital injection in dog under morphine-chloralose anesthesia and endotoxemia (endotoxin 1 mg/kg). The horizontal lines ndicate the aortic pressure levels of 100 and 0 mmHg.

basic anesthesia because it leaves the autonomic balance and reflexes largely intact (f 1960 Greisheimer 1965 Cox 1972 b) Admittedly the possibility exists that the obs responses reflect an interaction of pentobarbital and morphine-chloralose anesthesia. H ever the heart rate and blood pressure responses to pentobarbital (tachycardia and s hypotension) were similar to those it usually elicits in dogs without underlying morph chloralose anesthesia (Cox 1972 a). With these statements in mind, we believe our re warrant the following facets of evaluation

Control state The slight tachycardia in the control group of dogs was probably a r of an activation of the sympathetic nervous system, by the surgical trauma inherent in preparative procedures. The endotoxic dogs were in a state of shock as judged from basic heart rate, arterial pressure and acid-base balance (Ettinger and Suter 1970, Lebr Linden and Norman 1971)

Pentobarbital effects The small pentobarbital dose (8 mg/kg) injected, immediately pressed both the vagal and the sympathetic efferentation. The mean heart rate in the co dogs increased while a decrease in heart rate occurred in the endotoxic dogs. Like the h rate changes in these two groups, the arterial pressure changes differed as well. In control dogs a slight, though significant, fall was observed, whereas a marked drop en in the endotoxic dogs. The pulmonary arterial pressure of the former group slightly rose fell in the latter group. The aortic arch baroreceptor activity decreased equally in groups. Concomitantly the sympathetic efferentation was reduced and the respir arrhythmia was abolished. This set of events suggests depression of the baroreceptor r No significant changes were found in the atrial B-type receptor discharges and the left mean pressure.

The observed decrease of the vagal efferentation accounts for the tachycardia, a ty finding with pentobarbital anesthesia. This substantiates the earlier indirect eviden

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Thyrotropin-releasing factor Distribution in neural and gastrointestinal tissues

By

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Abstract

[illegible]päluoto, J F Koivusalo and R. Kraama. *Thyrotropin-releasing factor: Distribution in neural and gastrointestinal tissues.* Acta physiol. scand. 1978 *104* 175–179

[illegible] of several rat tissues were purified with methanol extraction, gel filtration and cation exchange [illegible]matography and measured for thyrotropin-releasing factor (TRF) concentration by radioimmuno[illegible] TRF immunoreactivity of neural tissues (hypothalamus, spinal cord, medulla, cerebrum and cere[illegible]) and gastrointestinal tissues (pancreas, stomach, duodenum and colon) behaved as synthetic TRF [illegible] the purification. This dual distribution suggests that TRF-secreting cells have their origin in the [illegible] neuroectoderm.

[illegible] words: TRF distribution, gastrointestinal, neural

[illegible]pothalamic release and release inhibiting factors or hormones are a group of small poly[illegible] which regulate the release of anterior pituitary hormones. Thyrotropin-releasing [illegible]tor (TRF) was first isolated and characterized as pGlu–His–Pro–NH from ovine (Burgus *et al* 1969) and porcine (Nair *et al.* 1970) hypothalamus. Later studies have demonstrated [illegible] presence of a TRF-like peptide by radioimmunoassay in extracts of other brain areas [illegible]d spinal cord, and in an immunocytochemical study TRF-positive material was located [illegible] spinal cord (Jackson and Reichlin 1974, Oliver *et al* 1974 Hokfelt *et al* 1975 Kardon *et al.* 1977). It has been postulated that all peptide hormone-producing cells are derivatives [illegible] the embryonal neuroectoderm and hence distributed in adults in the central nervous [illegible]stem, gastrointestinal tissues and skin (Pearse 1976). This postulate should explain why [illegible]bstance P somatostatin, gastrin, vasoactive intestinal peptide and neurotensin-like activity [illegible]ve been found both in the brain and gastrointestinal tract. In this report we have obtained [illegible]emical evidence that TRF-like peptide is located in various gastrointestinal tissues, and [illegible]nfirm our previous preliminary results (Leppäluoto *et al* 1977).

Material and methods

[illegible] Sprague-Dawley rats weighing 200–300 g were used. After decapitation specimens of following [illegible] samples (weighing ca. 1 mg–1 g) were removed: hypothalamus, pituitary, pons and medulla, spinal

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...I. Chromatographic characteristics of TRF immunoreactivity in purified rat hypothalamic and pancreatic samples. Results are means of 2 3 separate thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) runs. The following solvent systems ... used n-butanol, acetic acid and water (BAW 4 1 5), phenol and water (PW 3 1) and chloroform, methanol and conc. ammonia (CMA 5 3 1). For HPLC, 0.01 M NH_4OAc was used as starting buffer and then CH_3CN (0-100). The chromatographic mobility of hypothalamic and pancreatic TRF as measured in radioimmunoassay was in all the systems similar to that of synthetic TRF

...ple	Rf values in TLC			HPLC retention time (min)
	BAW	PW	CMA	
...othalamus	0.15	0.75	0.85	11
...reas	0.15	0.75	0.85	12
...reas plus ...F	0.15	0.75	0.85	12

Results

...F immunoreactivity in extracts of rat hypothalamus (Fig. 1 panel b), pituitary brain, ...nal cord, pancreas (Fig. 1 panel c), stomach, duodenum and colon was eluted in gel ...ration in LH-20 as synthetic TRF (Fig. 1 panel a), before the major part of the proteins ...d salts TRF immunoreactive fractions from gel filtration were submitted to SP C 25 ... the neutral 0.2 M buffer TRF immunoreactivity of the above-mentioned tissues was ...ted at 0.25-0.75 V_t, at the same volume at which synthetic TRF is eluted. In Fig. the ...tion of TRF immunoreactivity in hypothalamic and pancreatic samples (panel b and c) ... presented and compared with the elution of synthetic TRF (panel a).

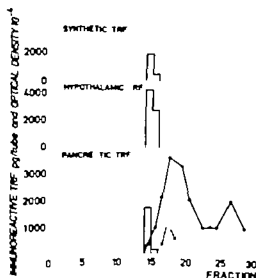
...LE II Distribution of TRF in various rat tissues. Each sample ...as extracted and submitted to gel filtration and cation exchange chromatography before radioimmunoassay Results are expressed as pg of TRF per mg of wet tissue (mean of 4-6 samples ± S.D.). Recovery of TRF was 90-100 ... Note high TRF concentrations in post. pituitary and spinal cord. There are also significant amounts of TRF in pancreas and intestine, but not in liver

...gan	Sample wet weight (mg)	TRF (pg/mg)
...ural tissues		
...ypothalamus	33 ± 10	410.0 ± 82.0
...st. pituitary	0.8 ± 0.1	380.0 ± 40.0
...nt. pituitary	8 ± 2	18.0 ± 5.0
...ons and medulla	275 ± 30	49.0 ± 15.0
...ervical spinal cord	67 ± 11	49.0 ± 7.0
...horacal spinal cord	60 ± 13	84.0 ± 23.0
...umbar spinal cord	65 ± 6	115.0 ± 43.0
...erebrum	1 170 ± 84	2.9 ± 0.8
...erebellum	300 ± 18	1.3 ± 0.5
...astrointestinal tissues		
...ancreas	434 ± 180	1.0 ± 0.1
...tomach	153 ± 21	0.5 ± 0.1
...uodenum	360 ± 61	0.4 ± 0.2
...olon	198 ± 28	0.2 ± 0.2
...iver	970 ± 82	0.03

cord cerebrum, cerebellum, Nervus trigeminus, N. sciatica, pancreas, stomach, duodenum, colon, muscle, lu g and kidney The tissue specimens were weighed and homogenized in 5 parts of 90% meth (w/v) or 1 ml at least. After centrifugation (10 min at 3 000 rpm) the supernatants were evapor 40°C under air and the residues were washed with 2 ml of 0.01 M acetic acid and chloroform. T phase was submitted to gel filtration in LH 20 (Pharmacia, Sweden, 1 × 50 cm column) in 100 met and 2–4 ml fractions were collected, evaporated, reconstituted in H_2O and measured for conduc optical density at 260 nm and for TRF in radioimmunoassay Our anti-TRF is specific to altera pGlu- and Pro-NH_2-moieties, but cross-reacts with His-modifications (Leppäluoto 1975, Ling et al Luteinizing hormone-releasing factor somatostatin, thyroglobulin and thyroid hormones do not react. The assay sensitivity is 1 pg.

TRF-containing fractions from gel filtration were submitted to cation exchange chromatograp SP C 25 (Pharmacia) column, (0.8 × 10 cm) equilibrated and washed with 0.01 M ammonium ace pH 5 7 TRF was eluted with 0.2 M ammonium acetate pH 7 4 The fractions were lyophil as before. The recovery of synthetic TRF (prepared by N Ling, the Salk Institute, U.S.A) after L and SP C 25 was 90–100%.

Partially purified hypothalamic and pancreatic samples were further submitted to thin layer chr graphy (Eastman Silica Gel No 6061) in 3 sol ent systems, butanol, acetic acid and ater (BAW 4 1 phenol and water (PW 3:1) and chloroform, methanol and conc. ammonia (CMA 5:3 1). After runs the sheets were sectioned into 5 mm regions, washed twice with 1 ml of 90% methanol, centr evaporated and measured in TRF radioimmunoassay Synthetic TRF was mixed with the half o pancreatic sample and used as control. Partially purified hypothalamic and pancreatic sample also submitted to *high-pressure liquid* chromatography (Varian, *U.S.A., CH-column*, 2.1 × 250 0.01 M ammonium acetate at pH 4 followed with increasing acetonitrile (0–100%) as described (Burgus *et al* 1976). Eluates were collected at 1 min intervals, lyophilized and measured in TRF r immunoassay



F g. 1

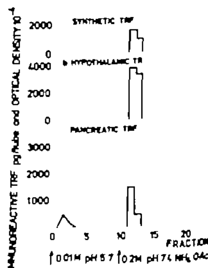


Fig. 2

Fig. 1 Elution profile of immunoreactive TRF extracted from rat hypothalamus (panel b) and pa (panel c). Column was LH 20, 1 50 cm, eluted with 100 methanol. 4 ml fractions were collecte measured for TRF proteins (optical density solid line) nd conductivity (i terrupted line). Elut synthetic TRF is presented i panel a. Not the simila elution of hypothalamic and pancreatic ex with synthetic TRF Other tissues (see T ble II) behaved similarly

Fig. 2 Cation excha ge chromatography of purified hypothalamic (panel b) and pancreatic (pa immunoreactive fractions from experiment presented i Fig. 1 Column was SP C 25 $V_t = 4$ ml and fractions were collected, lyophilized and measured in TRF radioimmunoassay (columns). The elut proteins (solid line) and synthetic TRF (panel) is also presented. Hypothalamic and pancreatic reactive TRF was eluted as synthetic TRF Other tissues (see T ble II) behaved similarly

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In TLCs and HPLC the R_f-values and retention times of TRF immunoreactive purified hypothalamic and pancreatic samples were similar to those in which synt. TRF was added (Table I) and only one immunoreactive peak existed.

Quantitatively (Table II) highest TRF immunoreactive concentration was fou. hypothalamus, 410 pg per mg of wet tissue, neurohypophysis had 380, spinal cord 84 medulla oblongata 49 and adenohypophysis 18 pg/mg. The extrahypothalamic brai cerebellum had 2.9 and 1.3 pg/mg respectively The immunoreactive TRF concentratio gastrointestinal tissues varied from 0.2 to 1 0 pg/mg, being highest in the pancreas, lo the colon and non-detectable in liver (less than 0 03 pg/mg) Muscle, kidney lungs and ripheral nerves (N sciatica and trigeminus) showed non-detectable TRF immunoreact (data not shown).

Discussion

In the course of this purification TRF immunoreactivity of hypothalamus, pituitary b spinal cord, pancreas, stomach, duodenum and colon behaved like synthetic TRF Hence reason that TRF belongs to the class of peptides, such as substance P somatostatin, ne tensin gastrin and vasoactive intestinal peptide (Pearse 1976), found both in brain and tissues. The cells producing these peptides may have a common origin in the embry neuroectoderm as we suggested before (Leppäluoto *et al* 1977) and also suggested r recent report in which TRF was found in the frog skin and retina (Jackson and Rei 1977)

Although the TRF concentration in gut tissues is low 0.2–1 pg/mg, the total TRF co in rat gut tissues is considerable 10–15 ng. This is comparable with the TRF cont found in hypothalamus or in brain The complex localization of TRF shown in this r lessens the clinical significance of the TRF measurements in plasma or in urine.

The main function of TRF in the Vertebrata is evidently the stimulation of TSH and p lactin release from the pituitary which would explain its high concentration in hypothala The extrahypothalamic distribution of TRF in brain suggests that TRF could operate neurotransmitter substance (Jackson and Reichlin 1974 Oliver *et al* 1974 Hökfelt e 1975 Kardon *et al* 1977) This suggestion is further supported by the widespread pharm logical effects of TRF Many of the functions which TRF affects are mediated via medul and spinal neurones and this might well be connected with our finding of a high medul and spinal cord TRF concentration. It is feasible that TRF would operate as a transm substance in the gastrointestinal tract too E.g. intravenous administration of synt TRF rapidly elicits abdominal sensations in human subjects and increases the intralum pressure of rabbit colon (Smith *et al* 1977). The role of TRF in these new locations rem to be established

After the submission of this paper we found that Morley and his collaborators have also shown immunoreactivity to occur throughout the rat gastrointestinal tract in *Bioch. m. Biophys. Res. C* 1977 79 314–318

We gratefully acknowledge the technical assistance of Tuula Pikkarainen, Seija Linnaluoto and Haataja. The English la guage was kindly revised by M Robert Brown.

I. Urine output (ml/hour) of subjects I–VII at control (C) and ethanol (E) experiment during four periods. N.S. = non-significant.

	6–9 p.m.		9–12 p.m.		0–6 a.m.		6–12 a.m.	
	C	E	C	E	C	E	C	E
	158	407	158	200	46	28	37	37
	337	490	57	153	13	28	50	37
	393	527	60	80	20	11	25	27
	300	307	83	40	3.	21	45	23
	150	333	73	143	20	20	20	20
	163	457	103	220	20	15	50	21
	180	92	103	777	23	57	50	57
m ± D	227±93	372±148	91±35	159±82	.3_11	26±15	40_13	32±13
	p 0.02		p<0.05		N.S.		N.S.	

...onized NaCl. Then, beginning at noon, they were kept in supine position for 22 h and fasted to 6 p.m. ...m 6 to 9 p.m. they received deionized water 10 ml/kg. Next morning at 8 a.m. they received water 5 ml/... At 10 a.m. they assumed standing position, walking slowly around. They received no food until noon. ...er that they ingested about 20 kcal/kg and 100 mmol sodium and 90 mmol potassium.

...beginning in the morning of the seventh day the same procedure as two days earlier was repeated, except ...t instead of water in the evening the subjects now received 15 % w/v ethanol 10 ml/kg. Blood samples ...re taken by venepuncture in control and ethanol experiment hourly in the evening from 6 to 10 p.m., at 12 p.m. and at 6, 8 and 10 a.m. and at noon. Urine was collected 6–9 p.m., 9–12 p.m., 0–6 a.m. and 2 ...m.

Plasma AVP was determined by radioimmunoassay (Fyhrquist *et al.* 1976). Blood ethanol and serum ...dium, potassium and chloride concentrations were measured as described (Ylikahri *et al.* 1974). In ...tistical treatments paired t test of Hewlett packard 9810 A calculator was used.

Results

During ethanol experiment urine output significantly exceeded the control value 6–9 p.m. ...d 9–12 p.m. (Table I). During the control experiment plasma AVP concentration showed ...urnal variation with higher values in the morning at 8 a.m. than in the evening at 6 p.m. (... 0.005). Physiological responses of AVP release (Fyhrquist *et al.* 1976) were seen in a ...t significant decrease of *plasma AVP in all subjects after water loading during the period* 10 p.m. as well as in an increase after getting up in the morning 10–12 a.m. ($p < 0.005$). Water loading suppressed plasma AVP near zero values in 3 subjects (I, II and V) between ... and 10 p.m., but there were large individual differences in the response of AVP to water ...he effect of slight water loading in the morning (8–9 a.m.) was significant during the ...hanol experiment (p 0.001), but not during the control one. Higher plasma AVP levels ...ere observed after 6 h of fasting while in a metabolic ward and on controlled sodium and ...otassium intake than in ambulatory subjects on free water supply (Fyhrquist *et al.* 1976).

During ethanol experiment a wide individual variation was seen in plasma AVP concentration. In subjects II, III and IV over 5 times higher AVP concentrations were observed ...uring ethanol intoxication than during the control period. These subjects also had the ...largest rise of plasma AVP after getting up at 10 a.m. *during hangover* and they were the

Acta physiol. scand. 1978. 104 180–187

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Plasma vasopressin in ethanol intoxication and hangover

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Abstract

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The effect of ethanol intoxication and hangover on immunoreactive plasma arginine vasopressin (AVP) concentration was studied in 7 healthy supine men in controlled clinical conditions. In 6 subjects plasma AVP increased above control values at the time of maximal blood ethanol concentration. The highest AVP values were observed in the subjects having nausea and vomiting and the worst hangover symptoms. During hangover plasma AVP values were higher than the controls and the response of plasma AVP to upright posture was exaggerated The dissociation of plasma AVP concentration and ethanol diuresis suggested that the suppression of AVP release is not the sole determinant of ethanol diuresis. The results may indicate that the toxic effects of ethanol and the severity of hangover symptoms are associated with the state of hydration and individual sensitivity of AVP triggering mechanisms.

The diuretic effect of ethanol is thought to be a consequence of suppressed antidiuretic hormone secretion (Wallgren and Barry 1970 Kissin and Begleiter 1971). However clinical and experimental data indicate that besides diuresis, ethanol may cause water retention (Wallgren and Barry 1970 Kissin and Begleiter 1971 Sargent *et al* 1975). Short-term experiments in humans (Flynn 1959) dogs (Millerschoen and Riggs 1969) and rats (Mar *et al* 1975 Linkola 1974) have even shown that after a transient diuretic phase antidiuresis soon follows.

Endocrine events relevant to the antidiuretic phase in ethanol intoxication and hangover have not been thoroughly investigated in man. Thus, there is no data on plasma antidiuretic hormone concentration during the whole period under the influence of ethanol. The present study describes immunoreactive plasma arginine$_8$-vasopressin (AVP) concentration during ethanol intoxication and hangover in supine man in controlled clinical conditions.

Materials and methods

7 healthy men, age 25–35 volunteered for the experiments performed under controlled clinical conditions. They received isocaloric diet (about 30 kcal/day/kg) containing 100 mmol sodium and 90 mmol potassium per day for 5 days. In the morning of the fifth day they received 2/3 the calories of that day and all ...

subjects (I, II, IV V and VII) and in ethanol experiment, 8-9 a.m., in all subjects. ...ld fluctuations in plasma AVP during 1 h periods between venipunctures could not be ...uded. This particularly refers to subjects III and VI, in which plasma AVP was not sup...ssed by water during the first hours of the experiment.

...revious reports (Wallgren and Barry 1970, Kissin and Begleiter 1971 Marquis *et al* ...5) have emphasized that the diuretic effect of ethanol is caused by AVP suppression. ...nitial diuresis has been thought to occur as long as blood alcohol concentration rises ...ggard *et al* 1941). In accord with this, we observed initial diuresis and a decrease of ...sma AVP during the phase of rising blood ethanol concentration in 5 subjects (II III, V and VI). Moreover plasma AVP increased above control values at the time of maximal ...od ethanol concentration in all but one (VI) subject, and paradoxal AVP peaks were ...erved in two subjects (III and IV).

...this study higher base-line AVP values were obtained than we have reported elsewhere ...hrquist *et al.* 1976). Such high basal AVP levels have not been observed in our later ...dies, either (unpublished results). Therefore, several possibilities should be considered, ...laining high basal AVP levels in subjects III and IV Fasting for 6 h, controlled sodium-...assium balance, warm summer during which the experiments were performed and the ...ss of being on a metabolic ward, *per se* may account for high base-line AVP levels. ...sible nonspecific interference of plasma factors cannot be retrospectively excluded. Such ...tors, however we have so far encountered in plasma from pregnant women only In ...ition in the particular assay in which all samples of subjects III and IV were assayed, ...h AVP levels may have been also caused by diminished immunoreactivity of AVP stan...ds. However, as it was the aim of this study to compare AVP values after ethanol inges... relation to control levels, this problem appears a secondary one. In fact, despite several ...oimmunoassay methods (Fyhrquist *et al.* 1976, Katz *et al.* 1976, Robertson *et al.* 1973) ... we yielded plasma AVP levels of roughly the same magnitude in man (0... 10 pg/ml), ...e are aware of no study giving the *absolute* values of AVP in human plasma at various ...els of hydration and in controlled clinical conditions.

In spite of higher AVP concentrations, initial ethanol diuresis exceeded control values in ... subjects (II and III). During the period 0-12 a.m. there were no significant difference ... urine output between ethanol and control experiment, in spite of higher plasma AVP ...ncentrations in ethanol experiment. Although specific evidence is lacking, some explana...ons for this apparent discrepancy also observed in subjects I, V and VII, and in mean ...ues, may be offered. Thus, catecholamines, the synthesis and release of which is stimulated ...uring ethanol intoxication (Wallgren and Barry 1970 Kissin and Begleiter 1971), may ...fect urine output. Noradrenaline has been described to counteract the cellular actions of ...sopressin at the level of cyclic AMP synthesis (Klein *et al* 1971 Beck *et al.* 1972), and ...opamine has been reported to increase renal blood flow glomerular filtration rate and ...dium excretion (McDonald *et al* 1964). Ethanol might also delay the event between ...VP receptor attachment and increase of luminal membrane water permeability in the ...ional collecting ducts, thereby causing a time lag between peaking plasma AVP values and ...antidiuresis. It is unlikely that ethanol would reverse AVP induced antidiuresis in man, ...because the long-acting AVP analogue, 1-desamino-D-arginine vasopressin (Ferring, Malmö,

TABLE II Serum sodium (Na), potassium (K) and chloride (Cl) concentration (mmol/l) before water or ethanol (E) ingestion (6 p.m.) and next morning (8 a.m.) and the corresponding means ±S.D Values of subject I are failing.

	[Na+]				[K+]				[Cl−]			
	6 p.m.		8 a.m.		6 p.m.		8 a.m.		6 p.m.		8 a.m.	
	C	E	C	E	C	E	C	E	C	E	C	E
II	140	141	140	144	3.9	4.0	4.3	4.3	106	106	105	[illegible]
III	140	140	141	145	4.0	3.8	4.7	4.7	104	104	102	[illegible]
IV	140	143	138	145	4.2	3.8	4.5	4.3	104	104	104	[illegible]
V	141	142	141	145	4.2	4.0	4.[illegible]	4.2	103	103	104	[illegible]
VI	141	141	140	143	3.6	3.9	4.3	4.4	105	105	106	[illegible]
VII	141	142	140	145	4.1	4.3	4.2	4.5	105	104	106	[illegible]
	141 ±0.6	142 ±1.1	140 ±1.1	[d]145 ±0.8	4.0 ±0.2	4.0 ±0.2	[b]4.4 ±0.2	4.4 ±0.2	105 ±1.1	104 ±1.0	105 ±1.5	[illegible]
	$p < 0.05$		$p < 0.001$									

differs not significantly from the value at 6 p.m.
[b] $p < 0.0$.., with respect to the value at 6 p.m.
$p < 0.005$ with respect to the value at 6 p.m.
[d] $p < 0.001$ with respect to the value at 6 p.m.

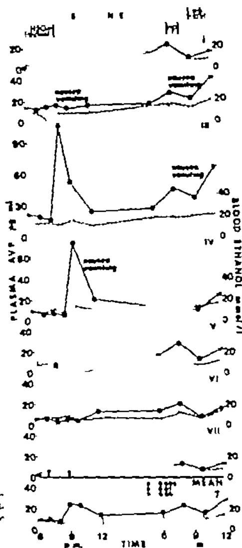
only subjects to have nausea and vomiting during the experiments. They also were the ones experiencing subjective hangover symptoms (Ylikahri *et al* 1974). Nausea and vomiting were temporally related to the AVP peak in subject IV but not in subject III. No clear AVP peak was observed in the case exhibiting the most serious signs of emesis (table II)

When ethanol and control experiments were compared subjects II and III had a discrepancy between plasma AVP and the diuretic response 6–12 p.m. Thus, antidiuresis did not occur despite increased AVP levels. Similar discrepancy was observed in subject I, 9–12 p.m., in subject V 9–12 p.m. and 0–12 a.m. and in subject VII 9–12 p.m. and 0–12 a.m. After midnight plasma AVP values were significantly higher in the ethanol experiment except after water ingestion in the morning. However no significant difference was observed in urine output between ethanol and control experiment between 0 and 12 a.m. The rise of plasma AVP was steeper during hangover 10–12 a.m. than during the corresponding control time.

Serum sodium and chloride concentration rose during ethanol experiment but not during control experiment (Table II). Serum potassium concentration increased in both control and ethanol experiment.

Discussion

Diurnal variation in plasma AVP was observed during the control experiment After overnight water deprivation, at 8 a.m., there were higher plasma AVP values than in the evening at 6 p.m. in accord with the data of Katz *et al* (1976). Possible secretory spurts as reported by Katz *et al* (1976) could not be distinguished because of the long time intervals between collecting blood samples. Water ingestion between 6 and 9 p.m. suppressed plasma AVP



1. Plasma vasopressin (AVP) of subjects I–... in control (O) and ethanol (●) experiment. ...ted line represents blood ethanol concentra... (not measured in I).

Thus, also alternative explanations should be offered for increased AVP response to ...anol. It has been concluded that potassium depletion stimulates thirst (Berl *et al.* 1977). ...uring water experiment subjects II, III and IV had the highest urine output between 6 and ... p.m. (Table I). This indicates high water intake while on the controlled diet. These factors ...d low initial serum [K] in subjects III and IV before ethanol ingestion (Table II) may ... dicate hypokalemia in these subjects (Berl *et al.* 1977). On the other hand subject VII ...d high serum [K] at 6 p.m. Sodium ions have been suggested to have a specific role ... AVP release and thirst (Andersson 1977). Present results may provide a parallel explana...on. Thus, potassium deficiency may as well have sensitized AVP releasing mechanisms. It ... tempting to hypothesize that the mechanism is basing on the function of Na –K ATPase,

Sweden) abolished ethanol diuresis (M M Nieminen *et al.*, to be published). In the experiment at 6 p.m subjects III and IV had the lowest serum [K+] (Table II). In a previous study (Linkola *et al* 1976) decrease of serum [K] was observed during ethanol intoxication In potassium depletion the renal response to vasopressin is blunted (Pribam 1970) This may partly explain co-existing high urine output and high AVP values.

Dehydration following higher urinary output after ethanol than after water ingestion (Table I) may physiologically explain high plasma AVP levels after ethanol. Increased serum [Na] (Wallgren and Barry 1970 Kissin and Begleiter 1971 Robertson *et al* and activated renin-angiotensin system (Linkola *et al* 1976, Kell *et al.* 1975, Farm Fabre 1975) may stimulate AVP release during ethanol intoxication. In animals, simultaneous administration of hypertonic sodium chloride solution and angiotensin II synergistic effect in stimulating AVP release (Andersson 1971). In subject III the increase in serum [Na] and plasma renin activity (reported elsewhere) were the most prominent during ethanol experiment This may indicate the role of renin-angiotensin system sodium ions in AVP release in subject III The reduction of blood volume following diuresis may also lower the threshold of osmoreceptors (Dunn *et al.* 1973). Blood pressure not monitored in this study and nausea may also have affected the results.

Among the most important factors regulating vasopressin release is serum osmolality (Robertson *et al* 1976, Hays 1976) which parallels blood ethanol concentration (R *et al* 1972, Robinson and Loeb 1971 Champion *et al* 1975). Osmoreceptors, though sensitive to hypertonic solutions (Hays 1976), could be stimulated by ethanol only react to raised tonicity which means the change of colligative properties of blood, by ethanol As ethanol diffusing freely into cells, is incapable of increasing effective pressure (Wallgren and Barry 1970), ethanol could hardly stimulate AVP release by d the osmotic pressure at osmoreceptive cells. Present knowledge of osmoreceptors ficient to permit further evaluation of this problem. It should be, however emp that ethanol doses used in this experiment may increase serum osmolality abo (Linkola *et al* 1976). The intra and extracellular effects of this increment, *per se* known but even the sympathetic stimulation after ethanol has been thought to be to the osmotic effects of ethanol (Regan *et al* 1966). Effects of ethanol on autonomic surrounding supraoptic nuclei cannot be excluded as a cause of AVP release, supporting data on this area is lacking.

High plasma AVP levels during hangover were probably due to dehydration, as by increased serum [Na] levels (Table II), and possibly to activated renin-ang system (Linkola *et al* 1976). During hangover the rise of plasma AVP in response to posture was exaggerated (Fig. 1). Lowered plasma volume due to dehydration increased the postural stimulus of upright position (Brown *et al* 1966)

Higher plasma AVP values in subjects II and III before ethanol ingestion (Fig. reflect their lower degree of hydration as related to the control experiment Suc degree of hydration may potentiate the effect of ethanol on plasma AVP because in I and VII having lower starting levels of AVP in ethanol experiment than in contro ment the response of plasma AVP to ethanol was weak. However initial serum subjects II and III did not indicate dehydration when related to the control value

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but this remains to be confirmed. Whether stimulating effects of ethanol on this ... were related to AVP release remains also to be studied. Potassium deficiency may ... have been partly responsible for high basal AVP levels and poor AVP response to ... in some subjects. Prostaglandins may also have contributed to high AVP values (Anderson 1977), as well as to co-existing diuresis and natriuresis in subject III, 6-12 p.m., in ... experiment, but supporting data is lacking.

While AVP release appears to be associated with nausea (Shelton *et al.* 1977) ... hardly explain high AVP in subject III (Fig. 1). Because also in subject II emesis was ... correlated to plasma AVP it is possible that at least during ethanol intoxication ... over there is no absolute association between nausea and vomiting and AVP release. ... is also supported by the findings of Husain *et al.* (1975). Decreased blood pressure, a ... ful stimulus for AVP release (Robertson *et al.* 1973), may occur during heavy ethanol ... toxication (Wallgren and Barry 1970) but was not studied in this work. On the other ... the moderate ethanol dose given did probably not cause blood pressure reduction suffi... to explain AVP release in our study.

Pronounced AVP increments after ethanol may thus reflect individual sensitivity of ... thalamic AVP releasing mechanisms. Subjects II, III and IV, having the largest differ... in plasma AVP between ethanol and control experiments, also were the most ... intoxicated ones (nausea and vomiting, Fig. 1) and had the worst subjective hang... symptoms. These associations, althought difficult to assess on the time axis, may be ... mon denominators of decreased alcohol tolerance, as indicated by emesis and hang... symptoms. To explain rised plasma AVP levels in ethanol intoxication and hangover, ... favor the principal mechanisms as previously discussed: 1. dehydration and the increase ... blood [Na] due to ethanol diuresis, 2. stimulated renin-angiotensin system, 3. mechan... associated with emesis or blood pressure decrease in some subjects and 4. rised osmol... of body fluids, 5. potassium depletion, prostaglandins or other factors, which were ... controlled here.

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ding the hormone releasing mechanisms in the pancreas. Our interest in such a possible control of pancreatic hormone release by vascular receptors originates from previous es on the plasma volume control during bleeding (Järhult *et al.* 1972, Järhult 1973 ult & Grände 1975, Järhult 1975 a, Järhult *et al.* 1976). These studies clearly indicated an important contribution to the plasma volume restoration after bleeding is made by cose-osmotic absorption of extravascular fluid into the blood stream, in turn due to a l increase in the plasma glucose concentration. The hemorrhagic hyperglycemia was d to be caused by a release of glucose from the liver and the effect seemed to be mediated several different links of the sympatho-adrenal system (Järhult 1975 b). With regard to pancreatic hormone release during hemorrhage, several studies have demonstrated that ding is associated with an increased plasma level of glucagon (*e.g.* Halmagyi *et al.* Järhult 1975 b, Lindsey Faloona & Unger 1975) and, in spite of the hyperglycemia, nchanged or even lowered plasma insulin concentration (*e.g.* Carey Lowerey & Cloutier Moss *et al.* 1970, Hiebert *et al.* 1973).

a recent publication we presented experimental data which indicated that the reflex one release in hemorrhage is elicited preferentially from arterial baroreceptors, whereas ial chemoreceptors and cardiopulmonary receptors play only minor roles (Järhult, nberg & Lundvall 1977). It therefore seemed reasonable to suppose that arterial baroeptors also may by reflex influence the secretory pattern of the islets of Langerhans, and present investigation was undertaken to test this hypothesis. Emphasis was placed on nalysis of the efferent pathways in such a reflex, since in recent years it has been shown the endocrine pancreas can be strongly influenced not only by adrenal catecholamines, also by direct sympathetic nerve fibres (Esterhuizen & Howell 1970, Marliss *et al.* 1973, Bloom, Edwards & Vaughan 1973 Porte *et al.* 1973 Bloom & Edwards 1975). eliminary data from this work has been published elsewhere (Järhult & Holst 1977 ult, Ingemansson & Holst 1977).

Methods

study was performed on 21 cats of both sexes (mean weight 3.8 kg). The animals were fed on normal t diet, but fasted 10–16 h before the start of the experiment. Anesthesia as induced with ether and ntained th mixture of chloralose (50 mg/kg) and urethane (50 mg/kg) given A tracheal cannula inserted and the animals breathed spontaneously throughout the expt.

Interference with afferent carotid baroreceptor activity. In all expts. interference with the normal afferent harge from the carotid baroreceptors was made 45 min after bilateral vagotomy in the neck. In 17 complete elimination of this afferent inhibitory influence was accomplished by bilateral section sinus nerve, which was dissected free 6–8 mm from the carotid bifurcation. In 4 other expts., the tid baroreceptor activity was changed by varying the distending blood pressure in isolated carotid s-glomus preparations. For this purpose, the right iliac artery was cannulated with polyethylene ng and the arterial outflow diverted to the two common carotid arteries via Harvard peristaltic p. The external carotid arteries were cannulated with polyethylene tubings about 1 cm distal to the tid bifurcation and the outflows diverted to funnel inserted into the right jugular vein. All other ral vessels in the vicinity of the carotid sinuses were ligated. Screw clamps were placed on the external tid arteries and on the right iliac artery. The intrasinusal pressure was recorded on each side with ham P 23 AC transducer connected to T-tube in the shunt close to the carotid bifurcation. With this nique, the mean distending pressure in the carotid sinus could be changed to the desired levels by adjustts of the pump and the screw clamps.

Interference with the efferent sympatho-adrenal system. In order to study the efferent adrenergic path-

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Reflex adrenergic control of endocrine pancreas evoked by unloading of carotid baroreceptors in cats

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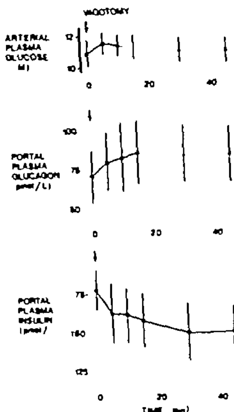
Abstract

JÄRHULT J and J J HOLST *Reflex adrenergic control of endocrine pancreas evoked by unloading of carotid baroreceptors in cats* Acta physiol. scand 1978 104 188-202

The effects of unloading of the carotid baroreceptors on arterial plasma glucose concentration and on portal plasma immunoreactive glucagon (IRG) and insulin (IRI) concentrations were studied in anesthetized, vagotomized cats either by sectioning the sinus nerves or by lowering the pressure in the carotid sinuses. Complete elimination of the carotid baroreceptor discharge by cutting the sinus nerves caused an increase in the arterial plasma glucose concentration by 100% and an increase in the portal IRG level by about 200% whereas the portal IRI concentration decreased to 50% of its basal value. The baroreceptor-induced changes of the plasma IRG and IRI levels seemed to be graded in relation to the drop in carotid blood pressure and they were clearly detectable when the pressure was lowered to 90 mmHg in the isolated carotid sinus preparation. The described reflex hyperglycemia, hyperglucagonemia and hypoinsulinemia were mediated to the pancreas and liver mainly by the sympathetic system, since cutting the splanchnic nerves above the adrenal glands abolished the hyperglycemic and hypoinsulinemic responses and markedly depressed the magnitude of the hyperglucagonemic response. In adrenalectomized cats, complete unloading of the baroreceptors evoked both hyperglucagonemia and hypoinsulinemia although the magnitude of the hormonal responses was diminished. In animals where the pancreas and liver were sympathectomized but the adrenal glands left intact, cutting the sinus nerves evoked a doubling of the IRG level and a slight increase in plasma glucose, but no significant change in the IRI level. I.v. infusion of adrenaline (1 µg/kg min) or noradrenaline (5 µg/kg min) caused pronounced increases in IRG and plasma glucose and a clear-cut reduction of IRI. We conclude that the function of the endocrine pancreas in the cat can be influenced by variations in the blood pressure by way of a reflex control which originates from arterial baroreceptors. This reflex adjustment of the endocrine pancreas is mediated chiefly by two links of the sympatho-adrenal system, namely by catecholamine release from the adrenal medulla and, more importantly by a direct adrenergic nerve fibre influence on the α and β-cells.

Key words: Baroreceptors, insulin, glucagon, glucose, sympatho-adrenal system

The literature on the physiology of baroreceptors is extensive (for reviews see Aviado & Schmidt 1955 Heymans & Neil 1958 Smith 1974 Kirchheim 1976). Yet there seems to be no report so far on a possible influence from the baroreceptors on metabolic



1 Effects of bilateral cervical vagotomy on ...al plasma glucose concentration and on por... ...tal immuno-reactive glucagon and insulin ...ntrations Data are given as mean values ± ... from 11 expts

...rial blood pressure (AP), heart rate (HR), arterial plasma glucose concentration and ...al plasma immunoreactive glucagon (IRG) and insulin (IRI) concentrations were fol...ed during 45 min. As can be seen from Fig. 1 vagotomy caused small but significant ...nges of IRG and IRI, whereas plasma glucose remained virtually unchanged. The IRG ...s increased from a control value of 70 ± 17 pmol/l to 85 ± 19 pmol/l 45 min after vago...y (p 0.02) and the corresponding IRI values were 177 ± 10 and 150 ± 18 pmol/l (p < 0.02), ...ectively The cardiovascular effects of cervical vagotomy consisted of a moderate, ...nificant increase in HR and an insignificant decrease in AP

...ovascular effects of complete elimination of the carotid baroreceptor discharge

...ting the sinus nerves evoked an immediate baroreceptor reflex consisting of tachycardia ... increased blood pressure (Fig 2). The heart rate rose immediately by about 50 beats/ ... and this level was maintained during the 45 min period of observation. AP showed a ...d peak response and then it gradually returned towards the control level. These reflex ...diovascular responses were seen in all animals, but the magnitude of the reflex responses ...ried somewhat between the 4 different groups of animals. The increase in AP was thus ... prominent in cats with pancreatic sympathectomy or in cats with sectioned splanchnic

ways in the baroreceptor induced adjustments of endocrine pancreas, the changes of glucose, and insulin concentrations in response to sinus nerve sectioning were followed after exclusion of links of the sympatho-adrenal system. In 5 cats the adrenal glands were removed bilaterally and care was taken to leave the direct sympathetic fibres to other regions intact ("Adrenalectomized cats"). In cats the sympathetic nerve fibres below the adrenal glands were removed by cutting all nerves along the celiac, mesenteric and hepatic arteries as well as in the mesenterium. In these expts. great care was taken to avoid damaging the preganglionic sympathetic nerves to the adrenal glands ("Cats with partial sympathectomy"). In 4 cats the major and minor splanchnic nerves were cut bilaterally and the [illegible] removed ("Cats with sectioned splanchnic nerves"). 4 cats with an intact sympatho-adrenal system served as controls ("Intact cats").

Cardiovascular recordings and blood sampling. After heparinization (1 000 IU/kg b.wt.), the right [illegible] artery was cannulated with a polyethylene tubing for pressure recording and sampling of arterial blood for plasma glucose determination. The arterial blood pressure (AP) was recorded with a Statham transducer and heart rate (HR) was registered with a tachograph triggered by the systolic pressure. A small jejunal vein was cannulated and a polyethylene catheter advanced until its tip [illegible] the portal vein. From this catheter blood samples were withdrawn for analysis of portal plasma immunoreactive glucagon (IRG) and insulin (IRI) concentrations. AP and HR were recorded on a Grass polygraph.

Hemorrhage. In order to investigate if hypovolemia could further augment the baroreceptor induced hyperglycemia, hyperglucagonemia and hypoinsulinemia, hemorrhage was performed in 4 cats with intact sympatho-adrenal system 45 min after sectioning the sinus nerves. The hemorrhagic hypotension was accomplished by bleeding the animal into a siliconized glass bottle connected via a T tube to the cannulated right brachial artery. The bleeding was rapid so that the arterial pressure reached 50 mmHg [illegible] min and this level was then maintained during 30 min with the aid of the pressure bottle.

Drug infusions. In order to compare the effects on endocrine pancreas of endogenously released catecholamines with those obtained by exogenous administration, adrenaline or noradrenaline was given i.v. 45 min after cutting the sinus nerves. The substances were dissolved in saline and infused at a rate of 0.5 ml/min in a dose of 1 or 5 μg/kg b.wt. × min for 10 min. Blood samples were drawn before, during and 35 min after the end of infusion.

Protocol. After completion of the surgery the animals rested for 30 min and then the vagus nerves were cut bilaterally in the neck. In 11 cats blood samples were drawn before vagotomy and then 5, 15 and 45 min after vagal denervation, whereas 10 cats rested 45 min after the vagotomy without sampling. The sinus nerves were then cut bilaterally in 17 cats and blood samples for glucose, glucagon and insulin analyses drawn before and 3, 6, 10, 15, 25 and 45 min after the sinus nerve denervation. In cats prepared with isolated carotid sinuses, the mean distending blood pressure of the carotid sinuses were lowered from the normal value (≈ 120 mmHg) to about 90 mmHg and then to about 60 mmHg. Each hypotensive period lasted for 10 min and blood samples were drawn at 3, 6 and 10 min.

After this observation period, hemorrhage to 50 mmHg was performed in 4 expts., adrenaline infusion for 10 min in 4 expts. and noradrenaline infusion for 10 min in 8 expts. Blood samples from the [illegible] and portal veins were drawn 3, 6, 10, 15, 25 and 45 min after the start of catecholamine infusion or 10, 20 and 30 min after the start of hemorrhage.

Biochemical analyses. Arterial plasma glucose concentration was determined on the autoanalyzer with a glucose-oxidase system (Trinder 1969). Portal blood was collected in ice-chilled tubes containing heparin (50 U) and aprotinin (Trasylol®, Bayer, GRF) 500 KIU/ml blood. Portal plasma insulin was determined according to Albano *et al.* (1972) and portal plasma glucagon concentrations by radioimmunoassay as previously described (Holst, Christiansen & Kühl 1976). The antiserum used is directed against the C-terminal sequence of glucagon and crossreacts less than 0.1 per cent with high concentrations of enteroglucagon. The detection limit of the assay is 5 pmol/l, the within-assay coefficient of variation is 6 per cent, and the inter-assay coefficient is 16 per cent.

Statistical methods. All data are presented as mean values ± S.E. The t-test for paired observations was employed for statistical evaluation.

Results

Effects of cervical vagotomy

Bilateral vagotomy in the neck was made in all expts. in order to facilitate the carotid baroreceptor response. In 11 of these expts. the effects of cervical vagotomy *per se* on sys-

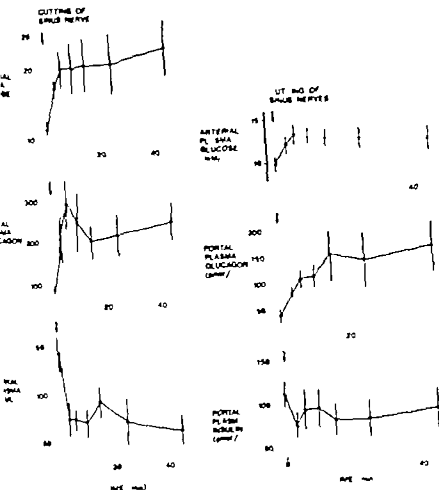


Fig. 3

Fig. 4

3 Effects of bilateral cutting of the sinus nerves on arterial plasma glucose concentration and on tal plasma immuno-reactive glucagon and insulin concentrations in vagotomized cats with an intact npatho-adrenal system. Data given as mean values ± S.E. (4).

4 Effects of bilateral cutting of the sinus nerves on arterial plasma glucose concentration and on tal plasma immuno-reactive glucagon and insulin concentrations in vagotomized, adrenalectomized Data are expressed as mean values ± S.E. (n   5).

rves and a similar quantitative difference was noted also with regard to the changes of art rate.

fects on glucose, glucagon and insulin of complete elimination of the rotid baroreceptor discharge

tact cats. Cutting the sinus nerves caused a characteristic response pattern consisting of perglycemia, hyperglucagonemia and hypoinsulinemia (Fig. 3). The arterial plasma glucose ncentration rose from   control level of 11 4 ± 1.6 mM to a maximum level of 22.5 ± 4.1

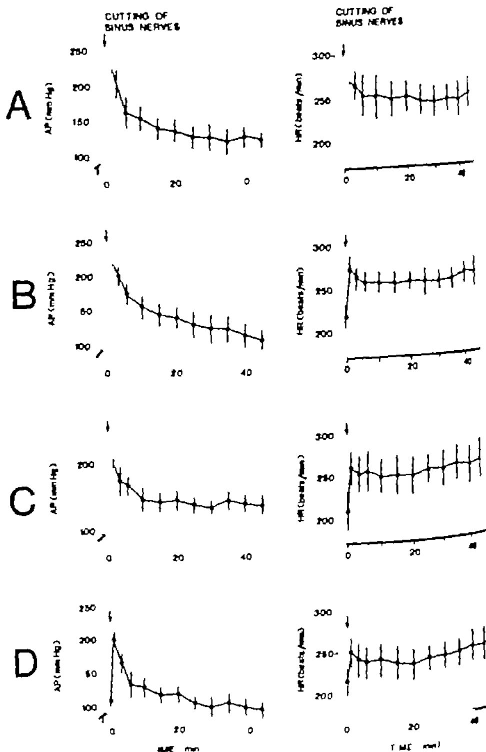


Fig. 2. Effects of bilateral cutting of the sinus nerves [illegible] vagotomized cats on arterial blood and heart rate (HR). Data are given as mean values ± S.E. Panel A shows the responses in (n = 4), panel B the responses in adrenalectomized cats ([illegible] = 5), panel C the responses in cats with sympathectomy (n = 4), and panel D the responses in cats with sectioned splanchnic nerves (n = 4).

I Effects on mean systemic arterial blood pressure (AP), heart rate (HR), arterial plasma glucose concentration, and on portal plasma glucagon and insulin concentrations evoked by stepwise reduction of the isolated carotid sinus pressure from about 120 mmHg dow to 90 and 60 mmHg, respectively The animals ere vagotomized in the neck about 45 min before experimental intervention. Data are expressed as mean values ± S E. from 4 expts.

	Carotid sinus pressure ~120 mmHg	Carotid sinus pressure ~90 mmHg			Carotid sinus pressure ~60 mmHg		
		3 min	6 min	10 min	3 min	6 min	10 min
mmHg)	126±6	192±16	171±19	165±19	183±19	156_13	130±18
(beats/min)	207±11	231 8	229±9	229±8	245±15	243±12	243±10
ose (mM)	11.8±1.3	13.1_1.5	14 2±1 6	14.4±1.5	15.9±1.5	17 1±1.7	17.5±1.6
agon (pmol/l)	176±14	284 44	331±95	335_90	366±112	418±97	449±136
lin (pmol/l)	77±23	51_14	51±15	49±14	41±12	47±18	48_21

to a similar degree in percent after sinus nerve sectioning. The IRI concentration raised 15–30% from control which is to be compared with the 50 % decrease observed he intact cats.

ats ith pancreatic sympathectomy As can be seen from Fig. 5, cutting the sinus nerves hese animals caused an increase in not only the concentration of IRG and arterial plasma cose but also of IRI. The glucose concentration rose by only 3 mM as a maximum, but concentration of IRG rose to a level twice that of the control values or from 68±25 ol/l to about 140 pmol/l. The increase in IRI was slight and not statistically significant.

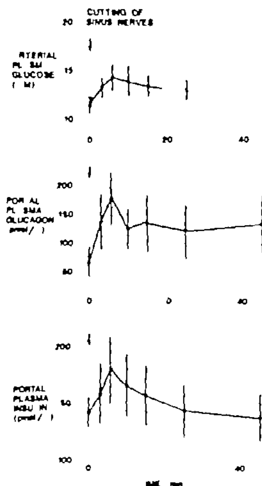
ats ith sectioned splanchnic nerves Fig. 6 illustrates that sectioning of the sinus nerves these animals did not significantly change the plasma glucose or IRI concentration. sma glucose tended t decrease gradually during the observation period and IRI to rease, *i.e.* the changes were opposite to those in the intact animals. However there curred a moderate increase in IRG from 60±13 pmol/l to about 130 pmol/l in response the complete baroreceptor unloading.

ffects on glucose glucagon and insulin of stepwise reduction of carotid pressure

the mean distending pressure of the isolated carotid sinus regions was reduced in steps m a normal AP of about 120 mmHg down t 90 and 60 mmHg, respectively it was found at there existed a close correlation between the mean distending pressure and the plasma els of glucose, glucagon and insulin (Table I). Clear-cut changes in hormone concentrans were observed even if the mean carotid pressure was lowered only to 90 mmHg, when e glucagon level was increased by about 100% and the insulin level depressed by about %.

ffects on glucose glucagon and insulin of hemorrhagic hypotension

4 cats with an intact sympatho-adrenal system, hemorrhagic hypotension to 50 mmHg as made 90 min after cervical vagotomy and 45 min after bilateral sinus nerve section. he effects on glucose, glucagon and insulin levels of this hypotension are depicted in Fig. 7 s can be seen, there was dramatic increase both in the arterial plasma glucose concentra-



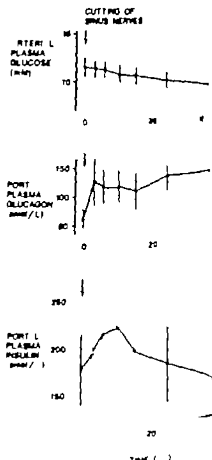


Fig. 5 Fig. 6

Fig. 5. Effects of bilateral cutting of the sinus nerves on arterial plasma glucose concentration and portal plasma immuno-reactive glucagon and insulin concentrations in vagotomized cats with ... sympathectomy. Data are given as mean values ± S.E. (n = 4).

Fig. 6. Effects of bilateral cutting of the sinus nerves on arterial plasma glucose concentration and portal plasma immuno-reactive glucagon and insulin concentrations in vagotomized cats with ... splanchnic nerves. Data are given as mean values ± S.E. (n = 4).

mM after 45 min, but a clear-cut increase was observed as early as 3 min after section of the sinus nerves. IRG rose from a control value of 88 ± 5 pmol/l to a peak value of 2... pmol/l after 6 min and it then remained virtually constant at a level 2–3 times above control. The control value of IRI was 143 ± 19 pmol/l but IRI very quickly dropped to about ... cent of its control value and the hypoinsulinemia was maintained during 45 min of observation.

Adrenalectomized cats. Fig. 4 illustrates that the adrenalectomized cats showed the same pattern of hormonal response to sinus nerve sectioning as the intact ones, though with some quantitative differences. Thus, the average glucose concentration increased only about ... mM in these cats; one cat, in fact, did not change its plasma glucose level at all. The I... concentration in the control period was lower in these cats than in the intact ones, be...

pancreas. As can be seen from Fig. 8, there was a considerable difference in the glucose agon and insulin responses to infusion of A and NA in a dose of 1 µg/min × kg b.wt. increases in glucose and IRG were thus much more pronounced for A than for NA with regard to IRI, infusion of A caused a clearcut decrease of IRI, whereas NA, if bling, tended to increase the IRI level. However an infusion of NA in a dose of 5 µg/ kg b.w.t. evoked a clear-cut increase in arterial plasma glucose concentration and al plasma glucagon concentration and a moderate decrease in portal plasma insulin centration, i.e. changes very similar to those elicited by A. — The cardiovascular onses of the two substances were very much the same.

Discussion

rious work in our laboratory has demonstrated that a significant part of the plasma ume restoration after bleeding is accomplished by a glucose-osmotic absorption of extra- ular fluid into the circulation, in turn due to an increased output of glucose from the r (see Introduction). In a recent publication we have presented experimental data sug- ting that this hepatic glucose release is caused preferentially by a reflex initiated from rial baroreceptors (Järhult, Holmberg & Lundvall 1977). Since hemorrhage is associated h increased plasma glucagon concentrations (Halmagyi *et al.* 1969, Järhult 1975 b, drey, Faloona & Unger 1975) and suppressed insulin release (Carey, Lowerey & Cloutier 0, Moss *et al.* 1970, Huebert *et al.* 1973), we found it worthwhile to investigate if the locrine pancreas also could be reflexly controlled from the arterial baroreceptors and, ther, if such a reflex could account for the observed hormone adjustments in bleeding.

We have found that complete elimination of the afferent baroreceptor discharge by sinus ve section evoked a distinct pattern of response in terms of hyperglycemia, hyperglucago- mia and hypoinsulinemia. Complete elimination of the baroreceptor discharge is equivalent a drop in arterial blood pressure to about 50–60 mmHg but the experiments with isolated rotid sinuses showed that a clear-cut increase in glucagon and decrease in insulin concen- ation occurred also when the arterial blood pressure fell from 120 down to 90 mmHg. ence, the adjustments of endocrine pancreas from carotid baroreceptors seem to be operating a ide physiological range of arterial blood pressure. The described response pattern is present also after adrenalectomy whereas cutting the pancreatic sympathetic nerves abolished the inhibiting effect on insulin secretion. Bilateral section of the splanchnic erves abolished both the hyperglycemia and hypoinsulinemia and diminished the hyper glucagonemic response. Exogenous catecholamines had effects similar to those observed uring sinus denervation in the intact cat. These findings indicate that the reflex is mediated the islets chiefly by the sympatho-adrenal system and that the adrenal catecholamines s well as activation of direct sympathetic nerves contribute to the full effect. The fact that certain increase of glucagon was present also after splanchnic nerve section might be taken indicate that the hyperglucagonemic response in part is due to a prolonged elimination the (see Lefebvre & Luyckx 1976).

A decrease of the portal insulin concentration was observed after baroreceptor-unloading the intact and in the adrenalectomized cat, but not in cats with pancreatic sympathectomy These data strongly suggest that the direct sympathetic innervation conveys an important

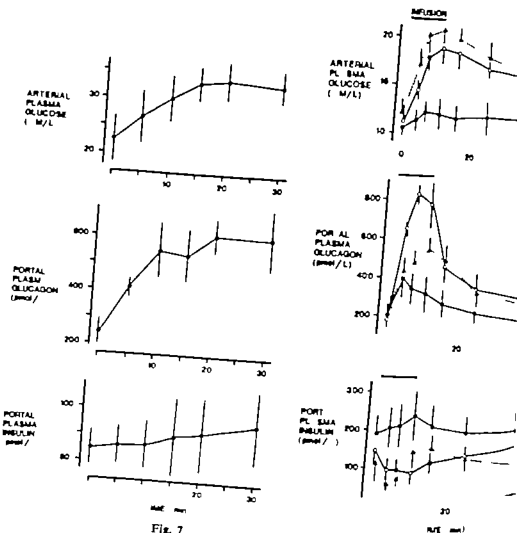


Fig. 7

Fig. 8

Fig. 7 Changes of arterial plasma glucose concentration and of portal plasma immuno-reactive glu and insulin concentrations in response to hemorrhagic hypotension to 50 mmHg. The hemorrhage performed 45 min after bilateral cutting of the sinus nerves in 4 cats with an intact sympatho-adr system. Data are given as mean values ± S.E.

Fig. 8. Effects of i.v. infusion of adrenaline (O—O) or noradrenaline (▲—▲) in a dose of 5 μg/min kg b.wt. or of noradrenaline (●—●) in a dose of 1 μg min b.wt. on arterial plasma glucose concentration and on portal plasma immuno-reactive glucagon and insulin concentrations. Data are given as m values ± S.E., n = 4 in each group.

tion and in the portal plasma IRG concentration in response to the bleeding, wherea portal IRI level was very little affected. As a maximum, plasma glucose increased fr 22.5 ± 4.1 to 33.8 ± 3.2 mM IRG from 245 ± 45 to 627 ± 128 pmol/l, and IRI from 62 to 85 ± 35 pmol/l. It should be noted that the control values at time zero in Fig. 7 are iden with the values obtained after 45 min in Fig. 3

Effects on glucose glucagon and insulin of exogenous catecholamines

Adrenaline (A) or noradrenaline (NA) were infused i.v. in 12 cats in order to get a rou comparison with the hormonal response evoked by reflex adrenergic activation of the end

the observed changes of portal insulin and glucagon levels most likely can be ascribed ...tered rates of hormone release.

...e present data on the glucose, glucagon and insulin concentrations taken together ...t that there is no distinct relation between the blood sugar level on the one hand and ...sulin, glucagon, or the insulin/glucagon ratio on the other. This is not unexpected in ... of the recent finding that the glucose-releasing effect of glucagon on the liver is not ...aled until the insulin concentration is depressed. Thus, at normal insulin levels, even ... physiological doses of glucagon given in the periphery or in the portal vein do not ...ase the blood glucose concentration (Sherwin *et al.* 1976, Felig *et al.* 1976, Holst *et al.* ...7). These observations are corroborated by our results. Hence, a pronounced glucose ...was noted in the intact and also in the adrenalectomized cats (Fig. 3 and 4), in which ...a the elevated glucagon was combined with a depressed insulin level. On the other ...d, in cats with cut splanchnic nerves (Fig. 6) a doubling of the portal glucagon level was ...ffective in raising the glucose level in face of a maintained portal insulin concentration. ...would also be noted in this context that the release of glucose from the liver is not only ...enced by pancreatic hormones but is also under effective control of hepatic sympathetic ...es and of humoral catecholamines from the adrenal medulla (Shimazu & Amakawa ...8 a, b, Edwards 1971, 1972 a, b, Edwards & Silver 1972, Järhult 1975 b). It should also ...borne in mind that the glucose response is quantitatively related to the amount of glycogen ...ed in the liver at the beginning of the experiment. Thus, animals with small glycogen ...es can develop much less of a hyperglycemic response to splanchnic nerve stimulation ...n animals with filled glycogen stores (Bloom & Edwards 1975). No glycogen determina...s were made in the present series of experiments, but it seems likely that the glycogen ...res were the same in all cats since they were all in the immediate postabsorptive state.

The problem whether the sympatho-adrenal system participates in the control of insulin ...d glucagon liberation during basal conditions is so far not entirely settled. Some authors ...ve reported that α-adrenergic blockers increase and β-adrenergic blockers decrease the ...sma insulin level in fasting unstrained man (Porte 1967) and in the unanesthetized mouse ...undquist 1972 a, b), but these findings in man could not be confirmed by Buse ... *et al.* ...970) or by Imura *et al.* (1971). Furthermore, the insulin and glucagon levels at rest are ...rmal both in sympathectomized and adrenalectomized man (Brodows, Pi-Sunyer & ...ampbell 1974, Järhult, Ingemansson & Holst to be published). The results reported herein ...ow that the control insulin concentration was higher and the control glucagon concentra...n lower in cats with cut splanchnic nerves than in those with intact nerves, which might ... taken to support a role for the sympatho-adrenal system in basal pancreatic hormone ...cretion.

The cardiovascular function of the carotid baroreceptors is to maintain the systemic ...lood pressure at a normal level by continuous adjustments of the heart and peripheral ...lood vessels. Since the brain is almost completely dependent on blood glucose for its energy ...quirements, it seems reasonable that the carotid receptors also should be able to control ...he release of glucose into the circulation, so that they in situations of cerebral hypotension ...nd hypoperfusion (such as in hemorrhage), can initiate hyperglycemia. The reflex increase ...n blood glucose concentration thus can compensate for the impaired cerebral blood flow

adrenergic influence on the β-cell in this reflex, whereas adrenal catecholamines shol of minor importance. This relative unimportance of the adrenal glands in the acute adjustments to stress has also been shown in a recent study by Bloom, Edwards & H. (1977). They found that only small amounts of catecholamines were released from the adrenal medulla even during severe hypoxia in the young conscious calf.

Our finding that direct sympathetic nerve fibres can play an important role in the control of endocrine pancreas is supported by previous experiments in which the splanchnic nerves or the mixed autonomic pancreatic nerve have been stimulated electrically (Porte huizen & Howell 1970, Marliss *et al.* 1972, 1973, Bloom, Edwards & Vaughan 1973, *et al.* 1973, Kaneto, Kajinuma & Kosaka 1975, Bloom & Edwards 1975; see also the review by Woods & Porte 1974). These studies have shown, in general, that activation of sympathetic nerves leads to a glucose and glucagon release but a roughly maintained insulin release. The relative maintenance of the insulin levels despite hyperglycemia has been interpreted as an α-adrenergic inhibition of the normal glucose induced insulin release. However, Kaneto and collaborators (1975) reported that the slight increase in insulin release in response to splanchnic nerve stimulation in the dog was abolished by pretreatment with propranolol, suggesting that the sympathetic nerves may influence both the α- and β-receptors of the insulin-producing cells. However, direct electrical activation is an unphysiological stimulus since it will activate both afferent and efferent axons belonging to the adrenergic, cholinergic and, perhaps, peptidergic system. Hence, the observed effects of electrical stimulation on insulin release will comprise the net sum of these different neurogenic influences on the β-cell. The present finding of a clear-cut decrease in the insulin concentration in portal blood may thus represent the true reflex adrenergic response of the β-cell, even if one may speculate that there exist selective nerve fibres which terminate on the α- or the β-receptors and that such fibres might be independently engaged during different reflexes.

The present investigation demonstrated that unloading of the carotid baroreceptors led to a 3-fold increase in the glucagon concentration and a 50 per cent decrease in the insulin concentration of portal blood. These changes indicate an enhanced release of glucagon and an inhibited release of insulin from the pancreas, providing the portal blood flow was not concomitantly changed by the baroreceptor reflex. Portal blood flow was not recorded in the present investigation, but previous studies on cats have demonstrated that the blood flow through different splanchnic organs, such as the small intestine and liver, remains virtually unchanged during complete unloading of the carotid baroreceptors, since the increase in vascular resistance is moderate and counterbalanced by the concomitant increase in perfusion pressure (Öberg 1964, Greenway, Lawson & Mellander 1967). Furthermore, the adrenergic vasoconstriction in splanchnic organs shows a characteristic "autoregulatory escape" phenomenon, implying that the vessels will dilate despite continuous adrenergic stimulation (Folkow *et al.* 1964, Greenway, Lawson & Mellander 1967). If pancreatic blood flow is mechanically reduced it seems that the insulin secretion is impaired only when pancreatic blood flow is reduced to 50% of its normal value for 40 min (Rappaport *et al.* 1971). It should also be noted that glucagon and insulin changed in opposite directions. It seems likely that the concentrations of the two hormones would have changed parallel to each other if the effects were merely caused by changed portal blood flow. It [illegible]

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so that the delivery of glucose to the brain remains relatively unchanged in these stre-
The relay station in the brain for this metabolic reflex has not been settled, but a ter
structure is the ventromedial part of the hypothalamus. Stimulation of this region has
shown to elicitate hyperglycemia, hyperglucagonemia and hypoinsulinemia (e.g. D
1953 Folkow & Euler 1954 Frohman & Bernardis 1971 Müller Frohman & C
1973 Frohman Bernardis & Stachura 1974).

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the glucose determinations.

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ferential effect on the sympathetic transmitter level in uterus and -er organs of guinea-pig by drugs interfering with adrenergic nerve functions

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Abstract

rbert, G P Alm, Ch. Owman, E. Rosengren and N -O Sjöberg. *Differential effect on the sympathetic transmitter level in uterus and other organs of guinea-pig by drugs interfering ith adrenergic nerve functions.* Acta physiol. scand. 1978. *104* 203–21..

es of adrenergic blocking agents with different modes of action were administered and the rate of nsmitter depletion as studied in different uterine regions of guinea-pig, and compared with that in the t and submandibular gland by combination of fluorescence histochemical and spectrofluorometric ods. The tyrosine-hydroxylase inhibitor, H 44/68, as well as reserpine and guanethidine, produced a efficient reduction in neuronal noradrenaline in the heart and submandibular gland than in the uterus. fferential action on the sympathetic transmitter, though less clearcut, was seen ith 6-hydroxydopamine. noradrenaline-depleting effect of metaraminol did not distinguish between those adrenergic nerves lying the uterus from those of the control tissues. It is suggested that the differential effects may be ed to lower activity in the uterine adrenergic nerve system, probably because this system is to large st composed of short adrenergic neurons, hich appear to have particularly slow transmitter turnover.

uterine adrenergic innervation undergoes dramatic alterations during pregnancy and hows a unique susceptibility to sex steroids as reflected in changes of the transmitter level man, Sjöberg and Sjöstrand 1974, Owman and Sjöberg 1976). In order to investigate the ctional and biochemical mechanisms underlying these changes, the guinea-pig has for eral reasons been chosen as suitable experimental animal model. On the basis of denervan studies, using histochemical and chemical analyses of the neurotransmitter the origin d distribution of the uterine adrenergic innervation of the guinea-pig have been described detail (Thorbert *et al.* 1977). The marked variation in the transmitter level occurring ring pregnancy and in the *post partum* period has been followed during narrow time tervals (Owman *et al.* 1975 Thorbert *et al.* 1978 a, Gårdmark, Owman and Sjöberg 1971), d has been possible to relate to alterations in transmitter synthesis as well as to degenerate and regenerative phenomena in the local innervation. These peculiar properties of the erine adrenergic innervation may at least partly be related t susceptibility of the nerves the action of sex steroids (*cf.* Thorbert, Alm and Rosengren 1978 b).

In the present paper certain functional properties of the uterine adrenergic nerves have been further elucidated by drugs known to interfere with various aspects of adrenergic function.

Materials and methods

Animals. Non-pregnant, sexually mature virgin guinea-pigs of the Dunkin-Hartley strain (400–500 g b.w.t.) were used. They were housed under standard conditions with regard to temperature, air humidity and light, and were given pellets (Astra Ewos, Sweden) and water *ad lib.* The estrous cycle was determined by daily inspection of the vaginal orifice. A smear was taken when the vagina was open. The smears were classified according to Sjövall (1938). The day of maximal cornification, before the influx of leucocytes in the smear, was called day 1 of the cycle. The animals were killed on days 8–12. An additional smear was taken on the day of sacrifice, and only animals with a clear leucocytic dominance were accepted for the study.

In vivo experiments. The animals received the following drugs: H44/68 (methyl ester of α-methyl-tyrosine, Hässle, Sweden), dissolved in saline, was given *i.p.* in a dose of 500 mg/kg followed by a new injection 8 h later. The animals were sacrificed 24 h after the first injection. Reserpine (Serpasil®, Ciba), diluted in 0.1 M citric acid, was given *i.p.* in doses of 0.1 and 0.01 mg/kg. The animals were killed 24, 48 and 96 h after the administration. Guanethidine bisulphate (Hässle, Sweden) was dissolved in saline. The amount administered was calculated as free base and was given in an *i.p.* dose of 30 mg/kg. The animals were studied 6, 12 and 24 h later. Metaraminol bitartrate, dissolved in saline and calculated as base, was given *i.p.* in a dose of 10 mg/kg, which was followed by another injection 4 h later. The animals were studied 12, 24 and 36 h after the first injection. 6-Hydroxydopamine (2,4,5-trihydroxyphenylethylamine hydrochloride = 6-OH DA) dissolved in ice-cold saline containing ascorbic acid (0.2 mg/ml) was given *i.v.* under light ethyl ether anesthesia into the saphenous vein in doses of 5, 10 and 20 mg/kg, and the animals were studied 4 h later.

The animals were sacrificed under a light ethyl ether anesthesia at the various time intervals after drug administrations, and tissue pieces were taken from the following organs: submandibular gland, heart (auricle–mainly left), and uterus (specimens from the suspensory ligament and the mid-portion of the uterine horn, as well as from the cervix).

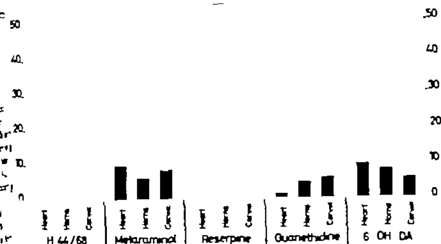
Neuronal noradrenaline was demonstrated histochemically by the fluorescence technique of Falck and Hillarp (for details, see Björklund, Falck and Owman 1972).

The fluorimetric method of Bertler *et al.* (1958), as modified by Häggendal (1963), was used in the quantitative chemical determinations of the amine in preparations consisting of the entire heart, uterine horns and cervix. The recovery of noradrenaline was 88% ± 3 (mean ± SE, n = 7). Correction for recovery was not made. The coefficient of variation was 3% for a pool of uterine tissue (n = 5) and 5% for a pool of cardiac tissue (n = 5). Because of a possible occurrence of α-methylated metabolites from α-methyl-tyrosine in experiments where H44/68 was given, the detectability of such metabolites (α-methyl-dihydroxyphenylalanine, dopamine and noradrenaline) was tested by adding these α-methylated compounds to tissue homogenates with known endogenous noradrenaline content. In no case were the fluorimetric readings significantly changed ($p > 0.05$) by the addition of the α-methylated compounds.

Statistics. It was found that the recorded noradrenaline values after the drug treatments and in control animals were beset with heterogenous variation as tested by Bartlett's test (Snedecor 1946). Following transformation of the measured noradrenaline values into their logarithms, variance heterogeneity was found to be non-significant. The transformed values were treated statistically in the analysis of variance using factorial coefficients (Snedecor 1946).

In vitro experiments. Tissue slices were prepared according to Hamberger (1967) from myometrial strips of the uterine horns and from myocardial tissue. Krebs–Ringer bicarbonate buffer containing 0.2% d-glucose, was used as incubation medium. The composition of the Krebs–Ringer medium was described by Hamberger (1967). The buffer was saturated with 95% O_2 and 5% CO_2, and the incubation flasks were regassed every 60 min. In the experiments with 6-OH DA the incubation buffer was supplemented with ascorbic acid (0.02 mg/ml).

The slices were collected in ice-cold incubation buffer immediately following dissection. The experiments were started by incubating the tissue slices in drug-free buffer for 20 min at 37°C to equilibrate the slices to this temperature, which was then used in the further incubation procedure. 6-OH DA, reserpine, guanethidine or metaraminol (see also above) were added to the incubation medium in 10^{-4}–10^{-3} M



[illegible]. Effect of various drugs on the total organ content of noradrenaline in the heart, the two uterine [horns] and the cervix of guinea-pigs. Mean noradrenaline content after the various treatments expressed [as per]centage of the mean value from 5 untreated, dioestrous controls is shown. The amount of drug given, [admini]stration route used, and the time interval between the (first) injection and sacrifice [w]as for the various [drugs]: H 44/68: 500 mg/kg i.p. twice, [w]ith 8 h interval, 24 h. Metaraminol: 10 mg/kg s.c. twice [w]ith 4 h [interv]al, 12 h. Reserpine: 10 µg/kg i.p. once, 12 h. Guanethidine: 30 mg/kg i.p. once, 6 h. 6-OH-DA, [illegible] g/kg [illegible] once, 4 h. Analysis of variance using factorial coefficients showed significant differences at [p <] 0.05 in the degree of noradrenaline reduction in heart vs. horns or cervix following treatment [w]ith [H44/6]8, reserpine, and guanethidine. Other comparisons gave no statistical significance.

[illegible] tions, and the tissue slices [w]ere incubated in the presence of the respecti[ve] drug for 10–120 min. [The effect of] incubation time and drug concentration was tested on material from 4 animals, and the various [experi]ments also included control tissues incubated in drug-free medium only. Immediately after the in[cuba]tion was finished (in the case of metaraminol this [w]as always followed by rinsing for 30 min in drug-[free in]cubation buffer), the tissue slices were frozen in propane-propylene cooled by liquid nitrogen, and [proce]ssed according to the method of Falck and Hillarp (see Björklund *et al.* 1972) for fluorescence histo[chem]istry. From every animal used, specimens from the myocardium and the uterus [w]ere also taken for [flu]orescence histochemistry [w]ithout previous incubation.

Results

[H 4]4/68

[Eff]ects in vivo. Treatment with the tyrosine hydroxylase inhibitor H44/68, during a 16-hour [per]iod resulted in a marked decrease in the number of the sympathetic nerves in the sub-[man]dibular gland and the heart. The remaining nerve terminals exhibited a moderately [dec]reased fluorescence. The effect of H44/68 on the uterine adrenergic innervation was [mu]ch less pronounced. This was reflected in the chemical determinations of noradrenaline ([Fi]g. 1), showing that 42 per cent of the total amount remained in the cervix and uterine [hor]ns and 22 per cent in the heart after this treatment. Statistical analysis showed that the [eff]ect on the heart was more pronounced than in the uterus ($p < 0.05$ Fig. 1).

[Meta]raminol

[E]ffects in vivo. The result of the administration of 10 mg/kg twice with 4 h interval was [re]corded 12, 24 and 36 h after the first injection. Only very few adrenergic nerves with a slight

fluorescence were seen at all time intervals, and the findings were the same in all examined

In accordance with the fluorescence histochemical observations, tissue noradrenaline markedly reduced to a similar extent in all preparations analyzed fluorimetrically (Fig

Reserpine

Effects in vivo Changes in the neuronal level of noradrenaline were examined at time intervals following blockade of the amine storage mechanism by reserpine (0 1 mg In the auricle and submandibular gland a marked decrease in the number of green-fluorescent nerves was seen already after 6 h, and only very few weakly fluorescent nerves were 6 h later At 24 h the fluorescence had disappeared in all nerves of these two structures fluorescence in the nerves returned progressively as seen 48 and 96 h after the injection, at 96 h almost a normal number and fluorescence intensity had been attained.

The reduction in the transmitter followed the same time-course in the uterus, but levels were clearly higher than in the auricle and submandibular gland except 24 h after injection when virtually no fluorescent nerves were seen (only in the uterine suspensory ligament did a substantial number of fluorescent nerves remain at this time interval cordingly a nearly complete restitution of the fluorescent network of adrenergic nerves seen already within 48 h after the injection

The difference in reserpine sensitivity could be confirmed in the chemical determinations in which the noradrenaline reduction produced by a dose of 10 μg/kg at 12 h was significantly more pronounced in the heart than in the tissues from the reproductive tract (p < Fig. 1)

Effects in vitro Incubation of myocardial and myometrial tissue in the presence of and 10^{-4} M reserpine resulted, after 60 or 120 min in a marked reduction in the intensity and number of fluorescent nerves. The extent of the reduction was the same in both tissues The yellowish fluorescence produced by reserpine in the tissue did not interfere with microscopic evaluation of the specific fluorescence in the adrenergic nerves.

Guanethidine

Effects in vivo Guanethidine is an adrenergic neuron-blocking agent which produces transmitter depletion from the postganglionic sympathetic nerves. Thus, 2 h after the injection there was a moderate reduction in the fluorescence of the adrenergic nerves supplying the submandibular gland and the heart auricle, whereas the nerve fluorescence in the uterus seemed unaffected At later time intervals (6–24 h after the injection) no or only few fluorescent adrenergic nerves remained visible in the auricle, submandibular gland, and uterine horns and cervix. The effect on neuronal noradrenaline was much less pronounced in the uterine suspensory ligament. The chemical determinations of noradrenaline showed a similar pattern of difference in the response to guanethidine, and factorial analysis showed that the amount of transmitter reduction in the reproductive tract was significantly smaller than that in the heart ($p < 0.05$ Fig. 1)

Effects in vitro Incubation for 60 or 120 min in the presence of 10^{-4}–10^{-3} M guanethidine

[illegible]red the adrenergic nerve fluorescence in both myometrial and myocardial tissue the [illegible]s seemed to be more pronounced in the latter tissue.

-DA

[illegible]s in vivo Most of the preparations taken for fluorescence microscopy were obtained [illegible] animals receiving 20 mg/kg of 6-OH DA. In the auricle and submandibular gland [illegible] was a prominent general decrease in the intensity of nerve fluorescence; remaining [illegible] were barely visible. The fluorescence microscopic picture was different in the uterine [illegible] and cervix: only a [illegible]ery small number of fluorescent nerves remained, but with a dis[illegible]y higher fluorescence intensity than in the cardiac nerves. The chemical determinations [illegible]ed a marked reduction in tissue noradrenaline. The extent of reduction was not signifi[illegible]ly different when comparing the different tissues (Fig. 1).

[illegible]wer doses of 6-OH-DA (10 and 5 mg/kg) were also tested. The reduction in tissue [illegible]drenaline was proportionally less marked at these dose levels. There was a tendency [illegible] less extensive relative fall in the transmitter of the uterine horns (cervix not measured) [illegible]pared to the heart, though the difference was not statistically significant.

[illegible]fects in vitro Ten minutes incubation with 10^{-4} M of 6-OH-DA slightly reduced the [illegible]rescence intensity of the adrenergic nerves in the heart and uterine tissue. After 10 and [illegible]min incubation almost no adrenergic nerves could be seen. A lower dose (10^{-4} M) of [illegible]-DA produced essentially the same changes, although less pronounced. The myo[illegible]rial adrenergic nerves seemed slightly more susceptible than the myocardial nerves to [illegible]ction of 6-OH-DA.

Discussion

[illegible] female reproductive tract of the guinea-pig receives a complex supply of adrenergic [illegible] (Thorbert *et al.* 1977), originating both in classical prevertebral sympathetic ganglia [illegible] in peripheral relays located in the uterovaginal region (Frankenhäuser's ganglia or [illegible]). The functionally unique properties of this innervation (Owman *et al.* 1974, Owman [illegible] Sjöberg 1976) have been elucidated in pharmacological experiments in which the sym[illegible]etic nerves of the heart (auricular tissue) have been chosen as reference. The effect of [illegible] steroids (Thorbert *et al.* 1978 b) and pregnancy (Owman *et al.* 1975, Thorbert *et al.* [illegible] a) on the uterine nerves of guinea-pig has recently been investigated in detail. In the [illegible]sent paper certain aspects of adrenergic neurotransmission have been studied using [illegible]gs that (a) block amine synthesis (H44/68) and (b) storage (reserpine), that (c) release [illegible] transmitter by different processes (metaraminol and guanethidine), and (d) produce [illegible]ve degeneration through chemical sympathectomy (6-OH-DA).

[illegible]he hydroxylation of tyrosine to dihydroxyphenylalanine is the rate-limiting step in the [illegible]thesis of noradrenaline (Levitt *et al.* 1965). α-Methyl-p-tyrosine is [illegible] potent inhibitor of [illegible]osine hydroxylase (Nagatsu, Levitt and Udenfriend 1964), and produces a marked reduc[illegible]n in tissue noradrenaline (Spector, Sjoerdsma and Udenfriend 1965). This inhibitor or its [illegible]thyl-ester H44/68 (Corrodi and Hanson 1966, Corrodi and Malmfors 1966) has been [illegible]ensively used to estimate the noradrenaline turnover under various conditions (*cf.* Brodie *et al.* 1966). In the present study treatment with H44/68 resulted in a marked reduction in

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...e female reproductive tract of the guinea-pig receives a complex supply of adrenergic ...es (Thorbert *et al.* 1977), originating both in classical prevertebral sympathetic ganglia ... in peripheral relays located in the uterovaginal region (Frankenhäuser's ganglia or ...ion). The functionally unique properties of this innervation (Owman *et al.* 1974, Owman ... Sjöberg 1976) have been elucidated in pharmacological experiments in which the sym... ...etic nerves of the heart (auricular tissue) have been chosen as reference. The effect of ...steroids (Thorbert *et al.* 1978 b) and pregnancy (Owman *et al.* 1975, Thorbert *et al.* ...) on the uterine nerves of guinea-pig has recently been investigated in detail. In the ...sent paper certain aspects of adrenergic neurotransmission have been studied using ...gs that (a) block amine synthesis (H44/68) and (b) storage (reserpine), that (c) release ... transmitter by different processes (metaraminol and guanethidine), and (d) produce ...ve degeneration through chemical sympathectomy (6-OH DA).

The hydroxylation of tyrosine to dihydroxyphenylalanine is the rate-limiting step in the ...thesis of noradrenaline (Levitt *et al.* 1965). α-Methyl-p-tyrosine is potent inhibitor of ...osine hydroxylase (Nagatsu, Levitt and Udenfriend 1964), and produces a marked reduc... ... in tissue noradrenaline (Spector, Sjoerdsma and Udenfriend 1965). This inhibitor or its ...thyl-ester H44/68 (Corrodi and Hanson 1966, Corrodi and Malmfors 1966) has been ...ensively used to estimate the noradrenaline turnover under various conditions (*cf.* Brodie ...*al.* 1966). In the present study treatment with H44/68 resulted in a marked reduction in

neuronal noradrenaline, the effect being more pronounced in the heart and submandibular gland than in the uterine tissue. Hence this may be due to a difference in the turnover of the total transmitter pool in terms of for instance, a lower noradrenaline release or a more efficient transmitter re-uptake in the nerves of the uterine tissue. A similar discrep. has been shown for the effect of H44/68 in male rats, in which the noradrenaline remained essentially unchanged in the vas deferens and seminal vesicle while a pron reduction was seen in the heart and submandibular gland (Swedin 1970). The musculature of the male internal genital organs are supplied entirely with short adrenergic neurons (Sjöstrand 1965) this class of nerves forms part of the innervation in the uterus (Thorbert *et al* 1977) Hence, a lower rate of turnover in the system of short adrenergic neurons may have contributed to the differential effect of H44/68 in the present experiments.

α Methyl-*p*-tyrosine is formed from the methyl-ester H44/68, *in vivo* (Corrodi and ... son 1966), and is then partly transformed into α-methyl-noradrenaline via α-methyl dihydroxyphenylalanine and dopamine (Maitre 1965 Carlsson, Mesch and ... 1968), The fluorescence yield of these α-methylated compounds in the formaldehyde fluorescence method (*cf* Björklund *et al* 1972) is approximately the same as that of noradrenaline. This would mean that, if anything, the fluorescence microscopic ... of the degree of reduction in neuronal noradrenaline after treatment with H44/68 ... been underestimated On the other hand it was found that addition of these α-methyl products to tissue homogenates does not interfere with the fluorimetric determination noradrenaline. Another aspect of this biotransformation of H44/68 is that α-methyl adrenaline may cause a depletion of authentic noradrenaline from the sympathetic ... Such depletion of noradrenaline from the sympathetic nerves was obtained with metar... which acts by displacement of noradrenaline from its storage sites after uptake into the (Andén 1964) This mechanism of action did not distinguish between the adrenergic in the uterine tissue on one hand and in the heart and submandibular gland on the ... This was confirmed in the chemical determinations showing an approximately equal of noradrenaline depletion in both types of tissues. It is therefore concluded that noradrenaline depletion by α methylated metabolites has not been the major mechanism in the experiments utilizing H44/68

It is generally accepted that the main effect of reserpine involves blockade of the ATP dependent uptake and storage mechanism of the amine granules (see Carlsson ... As a net result of the normal activity of the neuron, the noradrenaline level is reduced ... new storage granules are synthesized (Häggendal and Dahlström 1971 Giachetti and ... 1975). This means that experiments with reserpine provide a kind of indirect measure neuronal activity in the sympathetic system (Gillespie and McGrath 1974). According reserpine treatment produced the same pattern of differential noradrenaline reduction uterus and in the heart-submandibular gland as H44/68 The experiments confirm a ... observation on the female reproductive tract of rabbits (Nilsson 1964 Owman and Sj... 1967) that the interruption of the postganglionic adrenergic nerves in the uterus re... the rate of reduction in tissue noradrenaline, whereas stimulation of the hypogastric had the opposite effect. The male internal genital organs are also markedly resistant to action of reserpine (Sjöstrand and Swedin 1967 Dall and Evan 1974), which, in accord...

he reasoning above, would support the view that the contribution of short adrenergic ns may be responsible for a slower turnover of noradrenaline in the uterus compared er sympathetically innervated organs.

anethidine is a sympatholytic agent which, in acute experiments, produces adrenergic n blockade and also has a direct noradrenaline-depleting effect (*cf* Costa 1966, Abbs Dodd 1974). This type of interference with sympathetic nerve function also showed a icantly more pronounced effect in the heart tissue than in the uterus. There may be al explanations for this differential effect, one being that the noradrenaline pool avail- for immediate transmitter release is relatively smaller in the short adrenergic neurons Orden 1969 Swedin 1970, 1972).

OH-DA produces a chemical sympathectomy through a specific neurotoxic action on nergic nerve terminals (Thoenen and Tranzer 1968). Under the conditions applied in present experiments, 6-OH DA markedly reduced the noradrenaline content in all ns analyzed. It is notable that the guinea-pig seems to be more resistant to this neuro- amine than other commonly used laboratory animals such as the rat and rabbit (un- lished observations). Our data on 6-OH DA effects on the guinea-pig heart are con- nt with data presented by others (O'Donnell and Saar 1974 Bentley Shibata and Cheng 5). With the lower doses there was a tendency (though not statistically significant) to a e pronounced effect in the cardiac tissue, and fluorescence microscopy showed a slightly erent appearance of remaining fluorescent nerves in the uterus and heart. Whether this ects a different susceptibility in various types of sympathetic neurons is not possible to blish on the basis of the present material. It is notable, though, that noradrenaline in the deferens is relatively resistant to the action of 6-OH-DA compared to other sympatheti- y innervated organs (Jonsson and Sachs 1970), unless this is given in high, repeated es (Cottle and Nash 1974).

The extensively developed smooth musculature in the entire system of internal male ital organs is innervated by the special type of "short adrenergic neurons (Sjöstrand 5 Owman Sjoberg and Sjöstrand 1974 Baumgarten, Owman and Sjöberg 1975), which inate in the peripheral ganglia located adjacent to the effector organ. This predominant ply by short adrenergic neurons in the male made it possible to conveniently analyze rmacological and physiological differences between these and the "classical" sympathetic ves, which arise in para- and prevertebral ganglia. Using a number of approaches it has n found that the short adrenergic neurons constitute a functionally unique system f rves with several peculiarities recently listed and discussed by Baumgarten *et al.* (1975). his kind of short adrenergic neurons represents considerable contribution to the sym- thetic innervation of the guinea-pig uterus (Thorbert *et al.* 1977). The presently observed armacological differences in comparison with tissues innervated by conventional sym- thetic nerves resemble—though they seem t be quantitatively less marked—those found the male genital organs, and probably reflect this contribution of short adrenergic nerves the uterus. Another possibility is that the entire sympathetic innervation as such is peculiar the uterus, an organ with several properties that are fundamentally different from other mpathetically innervated organs (*cf* Sporrong 1976). Taking the results from the pharmaco- gical experiments together, they seem to indicate that the net rate of transmitter turnover

is comparatively low in the uterine adrenergic innervation including such transmitter properties as, e.g. release of only comparatively small transmitter quantities (cf. S... 1971) This assumption is supported by the results obtained in the incubation experiments in which the pharmacological differences were to a large extent eliminated, namely conditions when the postganglionic innervation in the uterine preparations had been separated from its normal functional connexions.

A factor that might have been of importance in determining the pharmacological di... on the various tissues examined is the blood flow through the organs, which may have influenced the tissue concentration of the drugs tested. There are several reasons to believe that blood flow differences have not been a primary factor in the present experiments: (1) the drugs have usually been given in amounts that are well above the minimum effective dose, (2) the difference in response between uterus on one hand and heart and submandibular gland on the other was not a uniform outcome, metaraminol and 6-OH DA being exceptions for reasons discussed above, and (3) there is evidence that the flow rate in the uterus is intermediary to those of the heart and salivary gland (Kopin, Gordon and ... 1965 Fischer Kopin and Wurtman 1964)

It is thus concluded from the various experiments that the adrenergic nerves of the ... have a low functional activity in the nonpregnant guinea-pig One probable reason for ... is that the short adrenergic neurons, with their slow turnover rate, form a prominent contribution.

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Effect of lowering of the temperature on the tissue levels of cAMP cGMP PGE and $PGF_{2\alpha}$ in spontaneously beating rat atria preparation

By

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Abstract

…antainen, J T Metsä-Ketelä and H. Vapaatalo *Effect of lowering of the temperature on the tissue levels of cAMP cGMP PGE and $PGF_{2\alpha}$ in spontaneously beating rat atria preparation*. Acta physiol. scand. 1978 *104* 213–216.

…e levels of cAMP cGMP PGE, and $PGF_{2\alpha}$ were measured in spontaneously beating rat atria prepara… at 20°C and 37°C. At 20°C the formation of cAMP increased in correlation with the markedly increased …litude and reduced frequency The other biochemical parameters were not significantly affected by the temperature.

…words Cyclic nucleotides, prostaglandins, isolated rat atria, temperature

…anges in the bath temperature alter the function of isolated heart preparations sub…ntially Cold environment slows down the heart rate and increases the amplitude, while …vation of the temperature has the opposite effect. These adaptive changes seem to be …tially compensatory and the work output of the heart (frequency amplitude) remains …e same Kunos and Szentivanyi (1968) claim that the adrenergic receptor function of the …art alters from β to type as the bath temperature is lowered. Amer and Byrne (1975) …late these receptor alterations t the tissue levels of cAMP and cGMP Because cyclic …cleotides and prostaglandins (PG) are anatomically and functionally interrelated in many …udies, we have measured both these biochemical parameters in relation to temperature …aptation of spontaneously beating rat atria preparation.

Material and methods

…ale Sprague-Dawley rats weighing 250–270 g were killed by dislocation of the neck, their hearts were …moved, atria excised, and the preparations were made in prewarmed, oxygenated Tyrode solution con…taining 1 000 IU/l heparin (Medica Ltd, Helsinki) The atria were incubated in 20 ml cuvette with con…

The results have been presented in before (Parantainen *et al* 1977)

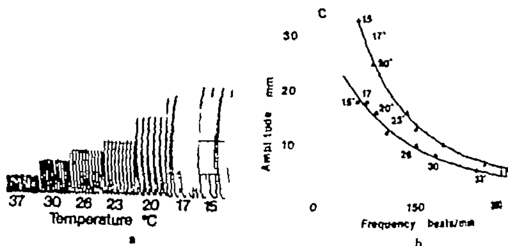


Fig. 1. Lowering of the bath temperature markedly decreases the heart rate and increases the amplitude. The changes are mutually compensatory and the work performed by the atria remains the same (b). ● are recordings from two different preparations.

tinuous flow of Tyrode (1.5 ml/min). Four preparations were installed parallelly and the mechanical responses registered isometrically by a Harvard Apparatus physiograph N:o 350. The basic tension was 0.5 g. After the stabilization of the preparation to 37°C or 20°C was completed (usually about ... min) the preparation was removed, clamped with tongs precooled in liquid nitrogen and homogenized with Braun Microdismembrator. The pulverized tissue was dissolved in 500 µl of ... (0 C) for determination of PGs, or correspondingly to 5 ... TCA for cyclic nucleotide assay. The sample was heated at 95°C for 1 min and stored ... −20°C for analysis. cAMP and cGMP were measured by protein binding methods of Gilman (1970) and Dinnendahl (1974), respectively. PGE (converted to PGB) and $PGF_{2\alpha}$ were determined by specific radioimmunoassays (Gershman et al. 1972, Jaffe and Behrman ... Jaffe et al. 1971. Levine and Van Vunakis 1970). Student's t-test was used in statistical comparisons.

Results

Lowering of the bath temperature to 20°C decreased the heart rate and caused a substantial increase in the amplitude (Fig. 1 a). The mechanical changes were mutually compensatory, the work performed by the preparation remaining the same at different temperatures (Fig. 1 b). The biochemical findings are summarized in Table I. Significant differences were obtained in the formation of cAMP. Compared to the level at 37° (5.24 pg/mg protein

TABLE I. Tissue levels of cAMP, cGMP, PGE and $PGF_{2\alpha}$ at tested temperatures.

	37°C		20°C		t-test
	$\bar{x}$	S.E. (n)	$\bar{x}$	S.E. ( )	
cAMP[a]	5.24 ±	0.44 (8)	6.55 ±	0.43 (7)	< 0.05
cGMP[a]	0.41 ±	0.03 (8)	0.36 ±	0.06 (8)	N.S.
PGE[b]	20 ±	3 (6)	23 ±	7 (8)	N.S.
$PGF_{2\alpha}$[b]	7 ±	10 (4)	31 ±	4 (4)	N.S.

[a] pg/mg protein.
[b] pmol/mg protein.

rmation of this nucleotide increased by 25% when the temperature was lowered to 20°C .05). The formation of cGMP did not change. Neither were there any significant altera- in the tissue levels of PGE or $PGF_{2\alpha}$.

Discussion

alterations in mechanical work performed by the atria at different temperatures are antial and, correspondingly should imply major changes in the biochemistry that lates the inotropic and chronotropic action. Yet no such changes were detected with d to cyclic nucleotides and PGs. The only significant finding was the elevation of cAMP h paralleled the increase in amplitude when the temperature was lowered. This is in rdance with the finding that cAMP mediates the positive inotropic action in adrenergic ulation (Robison *et al.* 1965 Vapaatalo *et al.* 1978). Accordingly Wollenberger *et al* .3) in ventricles of frog heart, and Brooker (1973) in isolated strips of frog heart demon- ted that in spontaneously contracting muscle the positive inotropic action was associated an increase in tissue cAMP concentration. The Wollenberger group also revealed that ering of tissue cGMP may accompany the rise in cAMP Similar cAMP–cGMP interac was observed in one series of our experiments with significant lowering of cGMP How the positive inotropic reaction may also be independent of cAMP (Øye and Langslet .9)

The relative insensitivity of PG formation to temperature changes was unexpected, as PG- thetase, the enzyme system that generates PGs, is reported to be sensitive to temperature ace-Asciak 1973). Furthermore, PGs are know to be able to modulate temperature aptation in some smooth muscle preparations (Strong and Chandler 1972) and they have od a capacity for regulation of local blood flow Compensatory mechanisms may be in- lved in maintaining the relative stability of the tested biochemical parameters. The adapta n of heart muscle to different temperatures seems to depend on other systems, the possible ndidates being other ATP-dependent enzymes, high-energy fatty acids and regulatory stems within the cell.

In conclusion, amongst the tested parameters only cAMP tended to parallel changes in ork output at different temperatures. The impetus for this correlation remains to be ucidated.

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Activity of acid hydrolases in skeletal muscle of untrained, trained and detrained mice of different ages

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Abstract

..öm, L., V VIHKO, E. ÅSTRÖM and A. U ARSTILA. *Activity of acid hydrolases in skeletal muscle of untrained, trained and detrained mice of different ages.* Acta physiol. scand. 1978, *104* 217–224

..tivities of p-nitrophenylphosphatase, β-glucuronidase and β-N-acetylglucosaminidase from crude .. muscle homogenates of 4 and 7 months old mice were assayed after short-term intensive and long-.. moderate training and after terminated training. In the older untrained mice the activity of the hydro.. was higher than in the younger mice. The level increased with training and this increase was far more .. nced in the older animals. Cessation of training for 7 and 21 days decreased this activity in the .. animals but it was again increased 42 days later and close to the level observed in the trained mice. In .. mice 3 days terminated training increased the activity of the acid hydrolases above the level of the .. animals but after additional 4 and 11 days' terminated training the activity decreased to slightly .. that of the trained mice. The changes were most prominent in the activity of β-glucuronidase and to .. extent in that of β-N-acetylglucosaminidase while p-nitrophenylphosphatase activity was almost un.. ..ed by training or terminated training. The effects of terminated training can be interpreted as represent.. ..lered catabolic processes in the turn-over of tissue components of skeletal muscle

..words Acid hydrolases, age, detraining, skeletal muscle, training

..activity of acid hydrolases in skeletal muscle occur normally and increased levels are ..ly reported in pathological conditions (Kar and Pearson 1972, Weinstock and Iodice ..). As their normal activity most probably contributes to regulating normal turn-over of ..lar contents, increased activity could be expected during periods of physical exercise. ..has been demonstrated that physical training induces higher activity of β-glucuronidase ..the skeletal muscle of mice, but does not significantly affect the activities of p-nitro..nylphosphatase or β-N-acetylglucosaminidase (Vihko *et al.* 1974). However this ob..vation was made on a rather small sample (five or six animals per compared groups), and ..order to study the effect more carefully the present investigation was undertaken. The in..tigation was performed with two types of training (intensive short-lasting and moderate

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I. Effect of age on the activities of acid hydrolases in skeletal muscle of mice

[illegible] basis	Enzyme	Activity		Difference between groups	t	P
		Younger	Older			
[illegible]	p-NPP-ase	6.456 ± 0.091 (27)	9.165 ± 0.159 (30)	42.0	14.3347	$< 10^{-4}$
	β-GU-ase	0.058 ± 0.002 (27)	0.102 ± 0.005 (30)	75.9	7.6243	$< 10^{-4}$
	β-GA-ase	1.599 ± 0.038 (27)	2.401 ± 0.081 (30)	50.2	8.6101	10^{-4}
[illegible] wet-weight	p-NPP-ase	1.026 ± 0.021 (27)	1.149 ± 0.022 (30)	12.0	4.0377	< 0.0005
	β-GU-ase	9.218 ± 0.396 (27)	11.361 ± 0.563 (30)	[illegible]3.2	2.7786	0.01
	β-GA-ase	0.255 ± 0.007	0.303 ± 0.011	18.8	3.5447	< 0.001

[illegible] activities of acid p-nitrophenylphosphatase (p-NPP-ase), β-glucuronidase (β-GU-ase) and β-N [illegible] glucosaminidase (β-GA-ase) in crude muscle homogenates of young (approx. 4 months) and older [illegible] 7 months) mice. The activities are expressed as nmoles p-nitrophenol formed per min either per [illegible] protein or per mg tissue wet-weight, except for β-GU-ase per wet [illegible] eight [illegible] here the activity is pmoles [illegible] of nmoles. The figures are mean values ± S.E. and the number of animals per group is given [illegible] [illegible] es. For further details: See Material and methods

II. Effect of training on the activities of acid hydrolases in skeletal muscle of mice

[illegible] basis	Enzyme	Age (months)	Activity		Increase in trained	t	P
			Control	Trained			
[illegible] protein	p-NPP-ase	4	6.456 ± 0.091 (27)	6.533 ± 0.096 (27)	1	0.5576	N.S.
		7	9.165 ± 0.159 (30)	9.783 ± 0.208 (28)	6.7	[illegible]3802	< 0.05
	β-GU-ase	4	0.058 ± 0.002 (27)	0.069 ± 0.002 (27)	19.0	3.2938	0.01
		7	0.102 ± 0.005 (30)	0.185 ± 0.024 (28)	81.4	3.5325	< 0.001
	β-GA-ase	4	1.599 ± 0.038 (27)	1.709 ± 0.041 (27)	6.9	1.9794	< 0.05
		7	[illegible]401 ± 0.081 (30)	3.301 ± 0.234 (28)	37.5	3.7280	< 0.001
[illegible] wet-weight	p-NPP-ase	4	1.027 ± 0.021 (27)	1.053 ± 0.018 (27)	2.5	0.9708	N.S.
		7	1.149 ± 0.022 (30)	1.225 ± 0.018 (28)	6.6	2.6729	< 0.01
	β-GU-ase	4	9.22 ± 0.40 (27)	11.13 ± 0.41 (27)	20.7	3.3314	< 0.01
		7	11.63 ± 0.65 (30)	20.23 ± 2.79 (28)	78.1	3.1962	< 0.01
	β-GA-ase	4	0.255 ± 0.007 (27)	0.276 ± 0.008 (27)	8.2	2.0039	< 0.05
		7	0.303 ± 0.011 (30)	0.415 ± 0.028 (28)	37.0	3.7907	< 0.001

[illegible] effect of physical training on the total activities of p-nitrophenylphosphatase (p-NPP-ase), β-glucu[illegible] ronidase (β-GU-ase) and β-N-acetylglucosaminidase (β-GA-ase) in crude homogenates from the skeletal [illegible] muscle of 4 and 7 months old mice. The enzymatic activities are expressed as nmoles p-nitrophenol formed [illegible] min per either mg protein or mg wet-weight of muscle with the exception of β-GU-ase per wet-weight, [illegible] here the activity [illegible] pmoles per min per mg. The figures denote mean values ± S.E. and within brackets [illegible] the number of animals per group. For further experimental details: See Material and methods

long-lasting). Furthermore, the investigation was extended to include the effects [illegible] minated training.

With this experimental design data was also obtained on the activity of acid hydro[illegible] the skeletal muscle of mice of different ages.

Material and methods

Animals

F_1-hybride mice of B6D2F1/BOM-strain (G. L. Bomholtgård Ltd, Ry, Denmark) were used for the [illegible] ment. At the beginning of the experiment their body weight was 27.1 ± 0.2 g (All data is given as mean ± S.E.) and their age 60 (range 53–67) days.

The mice were randomly divided into two main groups—either the younger group studied at the age of [illegible] weeks (113 ± 1 days) or the older group studied at the age of 30 weeks (208 ± 3 days). Within each group there were 3 subgroups, the first beign the untrained controls (C), which were living under [illegible] cage conditions with free acces to commercial solid food (Hankkija, Finland) and tap water ad li[illegible] second subgroup was the training group (TR), which ran on a motor-driven treadmill according to [illegible] programme (see below) up to the day they were killed. The third subgroup was the detraining or the (S) training group, which terminated the training at certain intervals before being killed (see below).

In the younger group the training of the two subgroups TR and S was very intense. T[illegible] by running for 60 min at a speed of 17.6 m/min daily (7 days a week) on the motor-driven treadmill [illegible] work load was increased to three periods of running per day (30 min at 20.5 m/min + 45 min at 17.6 [illegible] before noon and 75 min at 17.6 m/min in the afternoon). The training remained at this level for the TR group up to the day the mice were killed. The other subgroup (S) stopped training either 3, 7, or 1[illegible] before they were killed. During these periods they were caged in the same manner as the controls.

In the second main group—the older mice—the training of the TR and S subgroups [illegible] moder[illegible] lasted for 130–170 days. It consisted of running on the treadmill for 60 min at 20.5 m/min 5 days a wee[illegible] training remained at this level for the TR subgroup. The other subgroup stopped training 7, 21 or [illegible] before the day they were killed.

During the longer training period an increase in aggressive behaviour and higher mortality [illegible] served so that 28 of 107 mice died, mainly due to accidents on the treadmill or injuries caused by fi[illegible] with each other.

Enzyme assays

The mice were killed by dislocation of the neck, and the skin of the right leg was immediately removed, the calf cut off and chilled in ice-cold homogenization medium (see below). The muscle tissue was removed from bone and connective tissue as carefully as possible. After weighing, the muscle [illegible] genized in twenty time (weight to volume) of the homogenization medium. This was a solution of 1[illegible] KCl, 50 mM $KHCO_3$ and 6 mM EDTA, pH adjusted to 7.4 with HCl. Homogenization was performed [illegible] Potter Elvehjem homogenizer with a teflon pestle for 3 min. After homogenization all connective tissue which could be detected was removed and weighed. The remaining homogenate was used for the [illegible] enzymatic activities after the addition of Triton X 100 to a final concentration of 0.1%. The whole pro[illegible] tion procedure was performed at 0–4 C. Acid hydrolase assays were performed essentially according to Barret (1972) in the following way:

p-Nitrophenylphosphatase (acid phosphatase, E.C. 3.1.3.2) was estimated by incubating [illegible] homogenate together with 1.5 ml 0.1 M acetate buffer pH 5.0, and 0.5 ml 20 mM p-nitrophenylphosphate for 60 min at 37°C with 5 min preincubation before addition of substrate. After incubation 5.0 ml [illegible] NaOH was added and the extinction at 420 nm was estimated against a blank, in which NaOH was added before the substrate.

β-Glucuronidase (E.C. 3.2.1.31) was estimated by incubating 0.1 ml homogenate with 0.9 ml [illegible] acetate buffer pH 4.2. After 5 min preincubation 0.5 ml 5 mM p-nitrophenylglucuronide was added [illegible] incubation lasted for 18 h at 37°C. The reaction was stopped by adding 3.0 ml 0.4 M glycine buffer pH [illegible] After centrifugation the extinction at 420 nm was measured. A blank reaction was performed by ad[illegible] glycine buffer before the homogenate.

β-N-acetylglucosaminidase (E.C. 3.2.1.30). 0.1 ml homogenate was incubated together with [illegible] 0.1 M citrate buffer pH 4.8, 0.4 ml redistilled water and 0.5 ml 5 mM p-nitrophenyl-N-acetyl-[illegible]

a 37°C ...th 5 min preincubation before addition of the substrate. After 60 min incubation 2.0 ml NaOH was added and the extinction at 430 nm was measured against a blank in which NaOH was ... the substrate.

...protein contents of the homogenates ... as determined according to the method of Lowry *et al.* (1951) using 0.5 ml 2.0 ml 1 M NaOH at 40°C for 80 min and using bovine serum albumin for the standard ... in order to find out if there ... ere any differences between "tissue wet ... eight" and "protein content ... used for reference, the enzyme activities were calculated in both ways. The results were very similar ...-III) indicating that the treatment (training and detraining) did not induce edema or any similar ...ent.

...results ... are statistically compared by means of Student' t-test. All substrates were obtained from ... Chemical Co.

Results

...of age

...ctivity of the acid hydrolases was greater in the skeletal muscle of the older control ... than in that of the younger controls. The sizes of differences were not equal for the three ...ases but greatest in β-glucuronidase and lowest in p-nitrophenylphosphatase activity ...e I).

...ts of training

...ical exercise induced increased activities of the three acid hydrolases in the skeletal ...le of mice except p-nitrophenylphosphatase in the younger intensively trained animals ...le II). Thus β-glucuronidase activity increased approx. 80% in the moderately trained, ... and 20% in the intensively trained younger mice. β-N-acetylglucosaminidase activity ...ased 37 ... and 7%, respectively and p-nitrophenylphosphatase activity increased ...ox. 7% in the moderately trained group

...cts of terminated training

...itrophenylphosphatase was unaffected by detraining, although its activity resembled ... that of the trained animals (Table III, Fig. 1). The activity of β-glucuronidase was ...ificantly lower after training had been stopped for 7 and 21 days, respectively in the ... moderately trained group. After 42 days of detraining the activity of β-glucuronidase ...he 5 group increased and no significant difference was observed between it and either ... control or the trained groups. In the younger intensively trained groups the activity of ...lucuronidase was higher after 3 days of detraining than in the trained mice or in the ...trols. After 7 and 14 days the activity was at the same level as in the trained animals ...ble III, Fig. 1)

The activity of β-N-acetylglucosaminidase followed a similar pattern to that of β-glucuronidase in the younger, intensively trained animals, although the differences after 7 and 14 ...ys were not statistically significant. In the muscle of older moderately trained animals, this ...zyme had a higher activity than in the corresponding controls 7 days after training had ...minated. Fourteen days later the activity had lessened further and was between the two ...her groups. Fortytwo days after training had ceased the activity of β-N-acetylglucosaminidase had increased and was close to that of the trained animals (Table III, Fig. 1).

The duration of training and terminated training lasted for different periods in the younger ...d in the older animals and only ... period of 7 days detraining occurred in both age groups.

TABLE III A Effect of terminated training of different duration on the activities of acid hydrolases in skeletal muscle of old and young mice (per protein content).

Enzyme	Age (months)	Group	Activity at the end of 1st period	2nd period	3rd period
p-NPP ase	4	S	6.67 ±0.14 (9)	6.30 ±0.16 (9)	6.87 +0.3 [illegible]
		C	6.43 ±0.14 (9)	6.43 ±0.18 (9)	[illegible]
		TR	6.51 ±0.12 (9)	6.39 ±0.16 (9)	[illegible]
	7	S	10.30 ±0.29 (10)	8.85 ±0.17 (10)	10.17 ±[illegible]
		C	9 76 ±0.20 (10)	8.43 ±0.[illegible] (10)	9.30 ±[illegible]
		TR	10.37 ±0.36 (10)	8.75 ±0.24 (10)	10.21 ±[illegible]
β-GU-ase	4	S	0.070±0.004 (9)	0.069±0.004 (9)	0.075+[illegible]
		C	0 053±0.003 (9)	0.057±0 00[illegible] (9)	0.064+[illegible]
		TR	0.061±0.003 (9)	0.070±0.004 (9)	0.076+[illegible]
	7	S	0.113±0 006 (10)	0.101±0.007 (10)	0.184+[illegible]
		C	0.105±0 013 (10)	0.093±0 005 (10)	0.107+[illegible]
		TR	0.184±0.036 (10)	0.163±0 024 (10)	0.214+[illegible]
β-GA ase	4	S	1 711±0.053 (9)	1 741±0.072 (9)	1.773±[illegible]
		C	1.533±0 049 (9)	1 620±0.068 (9)	1.644±[illegible]
		TR	1.563±0 038 (9)	1 764±0.058 (9)	1.801+[illegible]
	7	S	2.799±0 146 (10)	2.517±0.109 (10)	3.789+[illegible]
		C	[illegible]351±0 [illegible]04 (10)	2.[illegible]61±0.083 (10)	2.591+[illegible]
		TR	3.095±0.293 (10)	3.108±0.284 (10)	3.802+[illegible]

TABLE III B Effect of terminated training of different duration on the activities of acid hydrolases in skeletal muscle of older and younger mice (per muscle wet-weight).

Enzyme	Age (months)	Group	Activity at the end of 1st period	2nd period	3rd period
p-NPP-ase	4	S	1.031±0.022 (9)	1.023±0.026 (9)	1.097±[illegible]
		C	0.982±0 040 (9)	1.039±0.036 (9)	1.080±[illegible]
		TR	1 021±0.015 (9)	1.015±0 033 (9)	1 124±[illegible]
	7	S	1 137±0 039 (10)	1 16[illegible]±0.028 (10)	1.281±[illegible]
		C	1 104±0 030 (10)	1 142±0 037 (10)	1.221±0[illegible]
		TR	1 191±0.036 (10)	1.209±0.020 (10)	1.289±0[illegible]
β-GU-ase	4	S	10.84 ±0 48 (9)	11 11 ±0.46 (9)	12.46 ±[illegible]
		C	8.17 ±0 58 (9)	9 18 ±0.36 (9)	10.30 ±0[illegible]
		TR	9 66 ±0.60 (9)	11 01 ±0.66 (9)	12.72 ±[illegible]
	7	S	12.19 ±0.55 (10)	9 05 ±0.48 (10)	21.54 ±5 34 [illegible]
		C	11 48 ±1 18 (10)	8.54 ±0.41 (10)	14 04 ±0[illegible]
		TR	1.07 ±4 03 (10)	14 49 ±1.90 (10)	[illegible]6.35 ±7 [illegible]
β-GA-ase	4	S	0.[illegible]65±0.010 (9)	0.282±0.010 (9)	0.292±0 012 [illegible]
		C	0.234±0.012 (9)	0.261±0 012 (9)	0.269±0 013 [illegible]
		TR	0.245±0.007 (9)	0.280±0 012 (9)	0.303±0 [illegible]
	7	S	0.307±0.015 (10)	0.334±0.014 (10)	0.460 [illegible] 0 [illegible]
		C	0 259±0 018 (10)	0.309±0.014 (10)	0.341±0 057 [illegible]
		TR	0.358±0.036 (10)	0.422±0 033 (10)	0.478±0 [illegible]

The effect of terminated training [illegible]n the total activities of acid p-nitrophenylphosphatase (p-NPP-ase), β-glucuronidase (β-GU-ase) and β-N-acetylglucosaminidase (β-GA-ase) in crude homogenates of skeletal muscle from 4 and 7 months old mice The enzymatic activities are expressed as nmoles p-nitrophenol formed per min per mg protei[illegible] (T[illegible]ble III A) or per mg muscle wet-weight (Table III B). β-GU-ase in Table III B is expressed as pmoles per min per mg muscle wet-weight.

1st, 2nd and 3rd periods denote in the 4 months group 3, 7 and 14 days after training had terminated. The corresponding periods in the 7 months group are 7 21 and 42 days. The group abbreviations refer to terminated trained (S-stopped), controls (C) and training (TR) mice. [illegible] and [illegible] denote statistically significant limits $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively when compared [illegible] S.

pothesis that aging is genetically governed has been proposed by Goldstein (1971), if it is true, might be the explanation for the high activity of the acid hydrolases.

of training

d training increases the activity of acid hydrolases in skeletal muscle and the increase pronounced in older mice than in young (Table II). Although the two groups were ed to different training programmes we are of the opinion that age has a greater in- on the increase than the intensity of training. The reason for this is that, in an earlier gation (Vihko *et al* 1974) with young mice trained in a similar way as the older mice study the increases in the activity of β-glucuronidase was equal to that found in the t younger group, which had trained more intensively It should also be noticed that in oup of older trained mice there were a few animals showing extremely high activities lucuronidase (and to lesser extent of β-N-acetylglucosaminidase) which increased the ing of the data (T ble II).

ronico and Bird (1970) have obtained results which suggest that the activity of β-glu- idase is partly derived from macrophages and connective tissue, while the activity of phosphatase (*Le* p-nitrophenylphosphatase) is mostly derived from the muscle cells. a bimodal distribution might be the explanation for the small or non-existing effect of ng on acid phosphatase activity in contrast to the marked effects on β-glucuronidase β-N-acetylglucosaminidase (Table II and III). However recent histochemical studies a and Gutman 1976) show the activity of all these three acid hydrolases also in muscle Thus the different effect of training on the activity of acid phosphatase and the two idases cannot simply result only in changes in cell populations and remains so far plained.

t of terminated training

kner *et al.* (1972) report loss of muscle fibres in giunea-pigs during detraining and iksson and Reitman (1977) report a reduction in the activity of succinate dehydrogenase cytochrome oxidase in humans during detraining. During such a reduction of activity cell components an increase of the activity of acid hydrolases would be expected. Only days of terminated training induced in the skeletal muscle of younger mice an activity of ucuronidase and β-N-acetylglucosaminidase which exceeded that in the younger mice under training (Table III, Fig. 1). This fits our original hypothesis. In all other groups two activities were not higher after termination of training than during training.

even days of terminated training gave different results for the two age groups. Thus in the r animals the activity of the acid hydrolases was similar in the control group and in the rained group but in the younger mice these levels were similar as between the detrained l trained mice. The activity of the older detraining group remained low for at least 21 days increased 42 days after training had stopped (Table III, Fig. 1). Activities of enzymes of rgy metabolism decrease after 5 weeks detraining in horses of respective age as our older but again after 10 weeks detraining a consistent but inexplicable increase in enzyme els was observed (Guy and Snow 1977).

It is possible that age causes a lag phase in the functions of catabolic mechanisms after

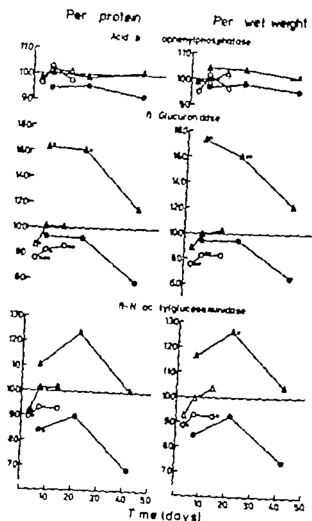


Fig. 1 The relative activities (...) of acid p-nitrophenylphosphatase, β-glucuronidase and β-N-acetylglucosaminidase ... trol (circles) and training mice ... compared to terminated training ... different periods after training ... Open symbols denote animals ... days and filled symbols denote ... 208 ± 3 days. Statistically significant ... tions from the values for the ... trained groups are indicated ... corresponding to $p < 0.05$, $p < 0.01$ ... $p < 0.001$ respectively. For further ... mental details See Material and ...

On this occasion, however the activities of the acid hydrolases were closer to those in the trained group in the younger animals and more similar to those of the controls in the ... group (Fig. 1)

Discussion

Age difference

The oxidative capacity of skeletal muscle fibre decreases with aging in guinea-pigs as shown in histochemical studies (Faulkner *et al* 1972, 1973 Maxwell *et al* 1973) The activity of acid phosphatase in different tissues of old mice—including skeletal muscle—is higher than in young animals (Youhotsky-Gore and Pathmanathan 1968). This is also true for β-glucuronidase and β-N-acetylglucosaminidase in skeletal muscle (Table I) Furthermore, the magnitude of the increase in activity of the acid hydrolases induced by training is greater in the old mice (Table II)

The difference caused by age in the oxidative capacity of skeletal muscle can to some extent be explained by the observed shift of the fibre composition in guinea-pigs (Faulkner *et al* 1972, 1973 Maxwell *et al* 1973) Whether this is also true for the acid hydrolases remains to be studied.

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…ts on fluid balance induced by non-febrile intracerebroventricular infusions of PGE_1, PGF and arachidonic acid in the goat

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Abstract

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…aglandins (PG) E_1, $F_{2\alpha}$ (30 ng/kg·min^{-1}) and arachidonic acid (150 and 300 ng/kg·min^{-1}) …ere in-…d for 30 min into the lateral cerebral ventricle of conscious hydrated and non-hydrated goats. Like …ously shown as concerns PGE_2, PGE_1 …as found to inhibit the …ater diuresis and cause some increase …renal sodium excretion in the hydrated animal, and to elicit thirst in the non-hydrated goat. The …ts of PGE_1 were enhanced …hen hypertonic (0.25 M) NaCl was simultaneously infused into the ven-… The antidiuretic effect of $PGF_{2\alpha}$ was less pronounced, and drinking only occasionally occurred …n this PG was infused into the non-hydrated animal. Only …eak, post-infusion reduction of the …ater …ass was observed …hen arachidonic acid …as administered into the hydrated goat. Neither the PG:s, …arachidonic acid affected the temperature regulation of the animals. The possibility is discussed that …lack of febrile response …as due to the choice of plasmon-tubing as material for the cerebral in-…sions. It is suggested that PGE_1 might have interacted …ith CSF Na … in stimulating juxtaventricular …ptors involved in the control of fluid balance. The experiments do not support the concept that PG …he E series constitute … cerebral humoral link in pyrogen-induced fever.

…ostaglandins (PG:s) of different types have been identified in various parts of the brain, …ong them the hypothalamus (Holmes and Horton 1968). It has been suggested that these …mpounds may be involved in several hypothalamic functions, such as regulations of food …take, anterior pituitary secretion and body temperature (*cf.* Wolfe 1975). That intra-…rebroventricular (IVT) or intrahypothalamic injections of PG of the E series produce an …evation of the body temperature has been demonstrated in several mammalian species, …e. the cat and rabbit (Milton and Wendlandt 1971) and the sheep (Bligh and Milton …973). In support of the idea that hypothalamic PG-synthesis is involved in the fever me-…anism is also the observation that IVT injections of the PG-precursor arachidonic acid …roduces fever in the rat (Splawinski *et al.* 1974).

That the hypothalamic regulation of antidiuretic hormone (ADH) secretion may be

terminated training, while in young animals the destruction in the muscle, including capillairs and connective tissue, starts immediately. A similar increase is not found in animals until 42 days after training has ceased.

This study was supported by grants (9290/79/77 and 11675/79/74) from the Ministry of Education, Finland and a grant from the Ellen and Artturi Nyyssönen Foundation. Mrs Arja Mansikkamäki provided technical assistance which is gratefully acknowledged.

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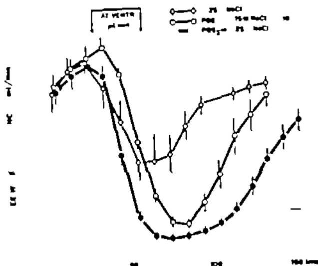
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A comparison between the ...retic responses to IVT in... s of hypertonic (0.25 M) ... and PGE_1 at isotonic or ...onic NaCl. The experiments ...performed in 4 hydrated ... Note the accentuation of ...sponse ...hen PGE_1 and ...tonic NaCl are simultane... administered. Dosage of ...30 ng kg min⁻¹. Vertical ... S.E.

Results

1. *Fluid balance*

...e effects obtained by the IVT infusion of PGE_1 were largely identical to those previously ...rved in response to the same dosage of PGE in the goat (Leksell 1976). As shown in ... 1, the IVT infusion of PGE_1 dissolved in isotonic (0.15 M) NaCl (n = 10) caused a ...ked inhibition of the water diuresis in hydrated animals. It implies that PGE_1 acted as ...efficient stimulus of ADH-release. This antidiuretic effect became much accentuated ...n the NaCl concentration of the CSF was simultaneously elevated. The infusion of ...E_1 in 0.25 M NaCl (n = 11) completely inhibited the water diuresis (= negative renal ...$_O$) for about 1 h, although the 0.25 M NaCl *per se* was not sufficient to induce negativ...o (Fig. 1). A similar PGE_1/Na interaction was seen as regards the renal sodium ex...tion in the hydrated animals (Fig. 2). The IVT infusions of 0.25 M NaCl and of PGE_1 ...otonic NaCl caused only minor increases in the sodium excretion, whereas a pronounced ...triuresis developed in response to the combined infusion of PGE_1 and hypertonic NaCl.

In the non-hydrated goat the IVT infusion of PGE_1 in isotonic NaCl (n = 5) invariably ...duced drinking. The average latency time for the dipsogenic response was 9 min and ...0 140 ml of water was consumed.

$F_{1\alpha}$

...he antidiuretic effect of $PGF_{1\alpha}$ was much less pronounced than that induced here by ...GE_1, and previously by PGE_1 (Leksell 1976). As shown in Fig. 3 the water diuresis gradually ...ecame reduced during the IVT infusion of $PGF_{1\alpha}$ in isotonic NaCl (n = 8) into the hydrated ...oat. However the infusions did not induce negative renal C_{H_2O}, indicating that only small ...mounts of ADH were released. These infusions had no appreciable effect upon the renal ...odium excretion.

affected by PG:s of the E series was originally suggested by Vilhardt and Hedqvist who found that intracarotid infusions of PGE inhibited the water diuresis of hydrated ... Accordingly in a later study (Leksell 1976) it was observed that IVT infusions of P... affected various aspects of the cerebral control of fluid balance. The substance was ... stimulate the release of ADH in hydrated goats, to elicit drinking in non-hydrated ... and to interact with cerebrospinal fluid (CSF) Na in augmenting renal sodium ex... and elevating the arterial blood pressure. Unexpectedly PGE did not induce fever ... goat in spite of the fact that the IVT infusions by all probability raised the PG-concentr... of the CSF way above pathophysiologically feasible levels. It has been suggested, ... that pyrogen-induced fever is mediated by hypothalamic PGE_1 synthesis in the cat (F... and Gupta 1973), and IVT administration of PGF_1 has been observed to cause fe... the sheep (Hales *et al* 1973). Therefore, it was of interest to extend the previous study ... goat to involve the latter two PG:s and arachidonic acid.

Methods

Animals. Four adult female goats (b.wt. 31–36 kg) were used. The animals were kept in metabolism ... where all experiments were conducted and where the goats had free access to hay and ... ater. The ... were maintained in positive sodium balance by receiving 6 g of NaCl added to a daily ration of ... grain mix.

IVT implantations and infusions. The goats had a special cannula system (Åkerlund, Andersson ... Olsson 1973) implanted under general anesthesia (pembutal) into one lateral ventricle about 14 d before ... experimental series were started. The cannula-material was platinum-iridium in order to minimize ... body reaction. In all experiments CSF was observed to drain out of the cannula on gentle compr... of the neck immediately before and after the infusions. Thus, free mixing of the infused solution ... CSF occurred in all experiments. The infusion rate was 10 µl/min and the duration 30 min. The ... time interval between each experiment was two days.

Hydration. Hydration was accomplished by giving 100 ml/kg of 38°C water by stomach tube in... rumen 90 min before the IVT infusion was started.

Urine sampling and analyses. Urine was collected in 10 min samples via a Foley catheter. Urine ... was determined by use of an "IL" 343 flame photometer and an Adv. Instruments Inc. osmometer ... used to measure urine and blood plasma osmolality. A plasma osmolality round 290 mosm/kg was ... during hydration, and this value was invariably used for calculations of renal free water clearance ... during hydration experiments.

Solutions used for the infusions. PGE_1 (crystalline) and $PGF_{2\alpha}$-tromethamine salt were generously ... plied by The Upjohn Company. The PGE_1 was dissolved in 99 % ethanol and was stored in samples ... mg (+20 µl of ethanol) at −20°C. Within one h before the infusion, 1 ml of 1.9 mmol Na_2CO_3 was ... to the sample of PGE_1. The $PGF_{2\alpha}$-tromethamine salt was dissolved in 0.15 M NaCl within 30 min ... the experiment. Arachidonic acid (Nu Chek Prep. Inc.) was obtained from the Dept. of Chemistry, ... linska Institutet. It was dissolved in 99 % ethanol and was stored at −20°C in a concentration of ... About 15 min before the infusions were started the dissolved substances were mixed with NaCl sol... of adequate volume and concentration to perform the following IVT infusions

1) PGE_1 (30 ng/kg min⁻¹) in 0.15 M NaCl
2) PGE_1 (30 ng/kg min⁻¹) in 0.25 M NaCl
3) $PGF_{2\alpha}$ (30 ng/kg min⁻¹) in 0.15 M NaCl
4) Arachidonic acid (150 ng/kg min⁻¹) in 0.15 M NaCl
5) Arachidonic acid (300 ng/kg min⁻¹) in 0.15 M NaCl

For comparison infusions of simply 0.25 M NaCl were also made. Three control infusions comb... ethanol/0.15 M NaCl (in the same proportions as in the PGE_1 and arachidonic acid experiments) ... made in the hydrated animal.

2. *Temperature regulation*

of the experiments (PGE$_1$ n = 10, PGF n = 5 and arachidonic acid n = 7) the rectal rature was measured 10 min before and after the infusions. Mean pre-infusion and fusion values were 39 1 ± 0 0 and 39 0 ± 0 1°C respectively The maximum temperature e observed after the infusions was 0.3°C (up- or downwards). Piloerection, shivering er febrile reactions were not observed in any of the present expts.

Discussion

appears to deserve particular attention in the present study is that neither IVT in- s of the tested PGs, nor of arachidonic acid influenced the body temperature of the . The hypothesis that prostaglandins are mediators of the febrile response to pyrogens locus within the preoptic/anterior hypothalamic region is supported from several es. Among the supports are the observations in almost all mammalian species tested r that PGs of the E series induce fever when administered either into the cerebral icles, or directly into brain tissue near the frontal border of the third ventricle (*cf* n 1974). Only two exceptions have been reported, the echidna and the goat. Injections GE$_1$ and E$_2$ int the lateral ventricle of echidnas were found ineffective in raising the temperature (Baird, Hales and Lang 1974), and infusions of PGE into the third lernon and Leksell 1975) and lateral ventricles (Leksell 1976) were not observed to e fever or any obvious activation of heat conserving mechanisms in the goat. The results rted here do not provide any support for the possibility that cerebral synthesis of some PG than PGE$_1$ would instead be the mediator of pyrogen fever in the goat. Of interest comparison are similar studies performed in a closely related ruminant species, the p. These investigations agree that PGE as well as PGE have a febrile action when in- ed into the lateral ventricle of the adult animal (Bligh and Milton 1973 Hales *et al* 1973). ever Pittman, Veale and Cooper (1975) have reported that such injections often do cause fever in the newborn lamb. Furthermore, the same authors recently (1977) did not ceed in producing fever by preoptic/anterior hypothalamic microinjections of PGE l PGE$_1$ in the adult sheep, although bacterial pyrogen often had this effect when in- ed at the same cerebral sites. Nonetheless, in that study it was confirmed that IVT in- ions of larger amounts of PGE induce fever The conclusions reached by Pittman *et al.* 77) were that the sheep may be able to develop fever without the central involvement of 3, or if PG does play a role, it may act within the brain at a site other than the preoptic/ terior hypothalamic region.

The previous goat and some of the sheep studies referred to above, together with the esent results provide substantial evidence against the concept that PG has a general im- rtance as an intermediary in the fever mechanism. However it remains an intricate pro- em why the IVT administration of various PGs and arachidonic acid did not induce ver in the goat, whereas this has been a rather consistent effect in other investigated adult ammals. It could hardly be a question of dosages since the amount of PG infused in the oat (approximately 30 μg in 30 min) was of the same order as that found to cause a con- iderable elevation of body temperature in e.g. the sheep (Hales *et al* 1973). Although a

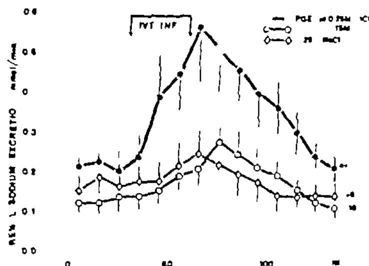


Fig. 2. Pronounced natriuretic response in the hydrated goat to IVT infusion of PGE_1 in hypertonic (0.25 M) NaCl. For comparison are shown the weaker effects of PGE_1 in isotonic saline and of solely the hypertonic NaCl. Dosage of PGE_1 ... kg min⁻¹. No of animals ... Vertical bars = S.E.

In only 2 out of 5 expts. performed in the non-hydrated goat did the animals drink during the infusion of $PGF_{2\alpha}$ dissolved in isotonic saline. Drinking occurred after 6 and 26 min; the amounts of water drunk were 700 and 140 ml respectively.

Arachidonic acid

No or little reduction of the water diuresis occurred during the IVT infusions of arachidonic acid in the hydrated goat, regardless of whether the large (n = 6) or the small dose (n ...) was administered. However, a temporary post infusion drop in the renal C_{H_2O} was observed which was most pronounced after that the large amount of the acid had been infused (Fig. ...). No obvious effect on the renal sodium excretion was observed in these expts.

Control infusions

The control infusions of solvent performed in hydrated animals (see methods) caused no reduction in renal C_{H_2O} or change in the renal sodium excretion.

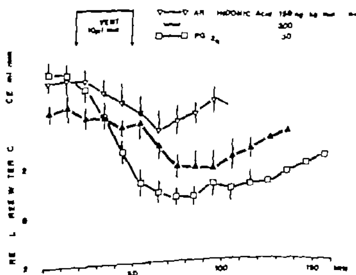


Fig. 3. Influences of IVT infusions of arachidonic acid and $PGF_{2\alpha}$ (in 0.15 M NaCl) on the course of the water diuresis in 4 hydrated goats. Note that $PGF_{2\alpha}$ has a considerably weaker antidiuretic effect than PGE_1 (see Fig. 1). Vertical bars = S.E.

... *Temperature regulation*

f the experiments (PGE_1 n = 10, $PGF_{2\alpha}$ n = 5, and arachidonic acid n = 7) the rectal ature was measured 10 min before and after the infusions. Mean pre-infusion and fusion values were 39.1 ± 0.0 and 39.0 ± 0.1°C respectively. The maximum temperature observed after the infusions was 0.3°C (up- or downwards). Piloerection, shivering er febrile reactions were not observed in any of the present expts.

Discussion

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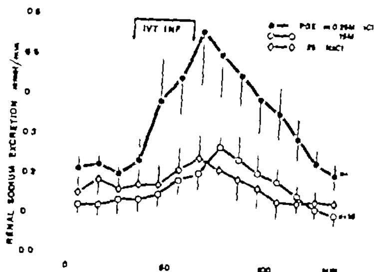


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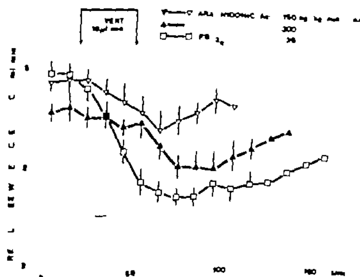


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species difference can not be excluded as the explanation, it appears more likely that particular experimental condition was the reason for the unresponsiveness of the [...] the present and previous PG experiments performed in this species, the permanent cannulae were made of a material causing very little foreign body reaction (plat-iridium), whereas stainless steel cannulae (more likely to cause deposits of metallic [...] been used in corresponding experiments performed in other mammals. The unique can-material might have been the ultimate reason why the goats did not respond with fe[ver to] the IVT infusions of PG:s and the PG precursor. If so, it implies that the fever elicited by IVT infusions in other species might have been secondary to PG-induced liberation of endogenous pyrogen from local inflammatory processes elicited as a foreign body re[action] to the cerebral implantations.

Juxtaventricular sodium sensitive receptors, the activation of which may be dep[endent] upon Na K ATPase activity, apparently play an important role in the cerebral con[trol of] fluid balance (*cf.* Andersson 1977). Earlier (Leksell 1976) it was found that the IVT ad-ministration of PGE, like elevated CSF [Na], stimulates thirst, ADH release, and [...] sodium excretion. It led to the suggestion that PGE_1 might affect periventricular [...] transport in a manner which stimulates receptors which primarily are sodium sensitive. [...] direct support for that idea was obtained by the previous observation that PGE [...] Na K ATPase activity (Limas and Cohn 1974). Also PGE_2 was then found to stimulate [...] Na^+-transporting enzyme, and was in the present study observed to affect the fluid ba[lance] of the goats in the identical manner as PGE. It appears possible, therefore, that PGE_1 [and] E_2 have the same action on juxtaventricular receptors involved in the control of fluid bala[nce.] The present experiments indicate that PGF and arachidonic acid are much less effective [in] that respect. However, the mere fact that IVT administration of PGE and E_2 are relatively efficient stimuli of thirst and ADH secretion can by no means be regarded [as] evidence that cerebral PG synthesis might play a physiological role in the control of [fluid] balance. The amounts of the substances needed to elicit these effects were obviously pharma-cological. A CSF concentration of PG in the order of 1 ng/ml has been found in [...] laboratory animals (*cf.* Hellon 1974). That concentration level must have been consider[ably] exceeded during the IVT infusions of PG performed in the goat.

This work was supported by the Swedish Medical Research Council (Project 04X-00503) and by [...] from Karolinska Institutet. I am indebted to Dr. Jan Svensson for invaluable discussions. The gen[erous] gift of PGE_2 and $PGF_{2\alpha}$ from The Upjohn Company is gratefully acknowledged.

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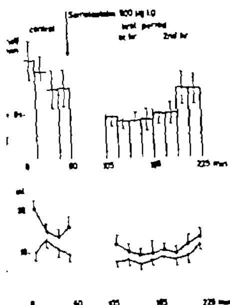


Fig. 1

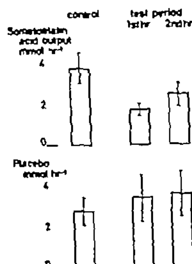


Fig. 2

1 upper Basal acid secretion (mmol/15 min) before and after intragastric administration of 500 μg somatostatin. Mean ± S.E. n=8. p 0.05.
er Volumes (ml/15 min) O—O and acidity (mmol/l) ●—● of basal gastric secretion. Mean ± S.E.

2. Basal gastric acid output (mmol h^{-1}) during control hour and following intragastric administration of somatostatin (n=8) or placebo (n=6). Mean ± S.E. p < 0.02.

4 0.63 mmol (p 0.02) and 1.26 ± 0.43 mmol (p 0.02) and the acid *output* was respectively 49.5 ± 9.6 and 69.6 ± 10.9 percent of that in control hour.

As a comparison (Fig. 2) placebo instillation did not change significantly the basal acid secretion which during the two test hours was 132.8 ± 28.6 and 135.1 ± 14.0 percent of that control hour.

1 000 μg intragastric somatostatin did not significantly change the gastric acid response to pentagastrin, the average acid output in 6 healthy subjects was 91.8 ± 6.8 and 106.9 ± 21.2 percent of control values during the first and second hour of stimulation.

Discussion. Somatostatin, first isolated from the hypothalamus was soon detected in the cells localized in the pancreas, stomach and in a decreasing number along the small intestine (Hokfelt *et al.* 1975 Polak *et al.* 1975). Somatostatin is a potent inhibitor of several secretory and motility functions in the gastrointestinal tract (Bloom *et al.* 1974 Bloom *et al.* 1975 Larsklsch *et al.* 1975 Johansson *et al.* 1978). These inhibitory effects might be due to blocking of the gastrointestinal hormone release (Boden *et al.* 1975 Bloom *et al.* 1975 Schlegel *et al.* 1977), but the gastric antisecretory effect is at least partly due to direct action on the parietal secretory cell (Barros *et al.* 1975).

Vagal stimulation releases somatostatin into the antral lumen of cats during acid perfusion, with concomitant depression of the gastrin release (Uvnäs-Wallensten *et al.* 1977).

Acta physiol scand. 1978. 104 232–234

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Effects of intragastrically administered somatostatin on basal and pentagastrin stimulated gastric acid secretion in man

By

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Given intravenously somatostatin is a potent inhibitor of gastric acid secretion, its effect being directed towards both the gastrin producing G-cell (Bloom *et al* 1974) and the parietal cell (Barros *et al* 1975) The physiological importance of somatostatin in the regulation of gastric acid secretion is unclear A role as a paracrine regulator of the pH-dependent gastrin release has been postulated (Uvnäs-Wallensten *et al* 1977). If somatostatin acts locally on the G-cell then intragastrically administered exogenous somatostatin might inhibit gastric secretion. This was tested by examining the effect of intragastric somatostatin instillation on basal and pentagastrin stimulated gastric acid secretion in healthy human subjects.

Methods Basal gastric secretion was collected in 9 healthy male subjects aged 21 to 29 mean 25 years. They were intubated after an overnight fast (Ryle tube 14 FG Portex). The position of the tube in the lower part of the stomach was controlled by fluoroscopy and the gastric residuum removed. With the subject lying on his left side and the saliva being withdrawn by a suction device, basal gastric secretion was collected by siphonage and gentle manual suction during four 15 min control periods. Thereafter was instilled 500 µg cyclic somatostatin (KABI Sweden) dissolved in 4 ml 0.15 M Na Cl. After washout of the tube with 1 ml saline and 10 ml air the subject turned round during 15 min, spending an equal period of time on each side of the body and was then allowed to walk around. At 42 min after the instillation, the gastric residuum was again removed, the subject lay down on his left side and gastric juice was collected in eight 15 min periods. One experiment was rejected, due to bile staining of more than two collections during the period and results from 8 expts. reported.

Placebo expts. with gastric instillation of 0 15 M NaCl performed in 6 subjects aged 22 to 29, mean years. The effect of somatostatin on pentagastrin stimulated gastric acid secretion was examined in 6 healthy male subjects aged 22 to 28 mean 4 years, who served as their own controls. The order of the expts. was randomised. The general procedure was as described above After complete aspiration of the stomach 500 µg cyclic somatostatin, or in controls, isotonic saline, was instilled through the gastric tube. An infusion of pentagastrin, 0 6 µg kg^{-1} h^{-1} (Peptavlon ICI) was started 45 min thereafter at a rate of 1 ml h^{-1}. Gastric juice was removed and gastric secretion collected in 15 min periods.

Volumes of the gastric secretion were measured and the acidity of gastric samples was determined by automatic titration with 0 1 M NaOH to pH 7 (Radiometer Copenhagen). Means are given ± SEM. The paired Student's t-test was used to test significance. In each individual test of the basal secretion the acid output during the control hour was compared with the first and second test hour Likewise was the mean acid output during the control hour compared with each 15 min collection period during the test period.

The study was approved by the Ethical Committee at Karolinska Hospital and informed consent was obtained from each subject.

Results Instillation of 500 µg somatostatin into the stomach of healthy subjects reduced significantly the basal acid secretion by depressing both volumes and acidity of the secreted gastric juice (Fig. 1) The reduction during the first and second test hour was on average

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[Cha]nges in muscle fibre type distribution in man after physical training

A sign of fibre type transformation.

By

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[Exp]eriments with cross-innervation and electrical stimulation of skeletal muscle in animal [(]Bárány and Close 1971 Pette and Schnez 1977) and man (Munsat *et al* 1976) indicate [the] possibility of transforming type I (slow twitch) to type II (fast twitch) muscle fibres and [vice] versa. Changes in the percentage of either fibre type related to physical training have not [bee]n reported in man. However endurance training decreased the type II B/II A ratio [wh]ereas immobilization increased this ratio (Andersen and Henriksson 1977 Jansson and [Ka]ijser 1977 Häggmark 1978). These observations indicate a reversible transformation [bet]ween type II A and II B fibres. A recent investigation on endurance trained subjects has [rev]ealed a high percentage of type II C fibres in the trained muscles (Jansson and Kaijser [19]77). These normally rare fibres have been assumed to be undifferentiated or intermediate [fib]res involved in reinnervation or motor unit transformation (Morris 1970, Kugelberg 1976). [Th]e significance of the elevated occurrence of type II C fibres in the well-trained subjects [me]ntioned above is difficult to evaluate but might be an indication of a progressing fibre type [tra]nsformation process. The aim of the present study was to throw further light on questions [co]ncerning fibre type transformation by studying long-term effects of different types of [ph]ysical training on the percentages of different fibre types.

Four long-distance runners participated in the study Mean values of age, weight and [he]ight were 21 (17–28) yrs, 67 (61 70) kg and 179 (174–187) cm respectively Maximal oxygen [u]ptake averaged 74 (71 77) ml kg^{-1} min^{-1} Their training activity could essentially be [di]vided into tw[o] periods. 1 "Aerobic" endurance training (18 (13–20) weeks), *i.e.* long-[d]istance running with an intensity corresponding to the "aerobic–anaerobic threshold level" [a]s defined by Mader *et al* (1976) (70–80% of VO_2 max.). The cumulated distance per week [w]as 110 (78–160) km, 2. "Anaerobic" training (11 (7–13) weeks) was mainly performed as [i]nterval training 2 3 times a week on track at 90–100% of their VO_2 max. This training re[s]ulted in high blood lactate concentration 14 0 (11 6–15 4) mmol/l. The cumulated distance [p]er week, which also included distance running, was 71 (40–118) km per week. Needle [b]iopsies were taken from vastus lateralis after both training periods. For subjects 1 and 2 the first biopsy was taken after the "aerobic" and the second after the "anaerobic" training [w]hereas the order was reversed for subjects 3 and 4 Serial sections of the biopsies were stained

Based on this finding and the close vicinity of the somatostatin producing D-cell and the G-cell in the antral mucosa a paracrine action has been postulated, implying that somatostatin may regulate the gastrin release by local action on the G-cell (Uvnäs-Wallensten et al.

If somatostatin exerts actions during and after its release to the gastric lumen, these actions should expectedly be reproduced by intragastrically instilled exogenous somatostatin. The reduction of the basal gastric acid secretion recorded in this study after intragastric administration of somatostatin supports this possibility. That this inhibition is due to a local effect on the antral G-cell has however to be confirmed by determination of gastrin levels after intragastric somatostatin. It should also be considered that these effects might be mediated by small amounts of somatostatin absorbed into the circulation since i.v. doses less than 30 μg/h are followed by a significant reduction of the gastric secretion (Johansson 1978 unpublished data).

In contrast to the inhibition of the basal gastric acid secretion, intragastric somatostatin did not inhibit the pentagastrin stimulated secretion, indicating that the peptide has, as administered had no direct effect on the stimulated parietal cell. The present data are, however, inadequate for the exclusion of such a possibility which has to be further tested with somatostatin doses instilled into the fundic part of the stomach.

The gastric antisecretory effects of intragastrically administered somatostatin may have clinical implications provided that the hormone, when given by this route, is not absorbed in concentrations known to suppress the release of a series of different hormones like insulin, glucagon, growth hormone and other. Such a broad spectrum of effects of intravenously given somatostatin is the principle obstacle for its introduction into clinical medicine.

Supported by grants from the Swedish Medical Research Council no. 19X 580.

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ng similar to type I fibres (II C') to fibres staining similar to type II A fibres (II C"). To n the finding that the percentage of type II C fibres was increased after "anaerobic" ng but not after "aerobic" training one must assume that the reversible transformation ss between type I and type II C is faster than the reversible transformation process een type II C and type II A fibres. Our findings concerning the possible transformation type I to type II fibres were supported by recent observations on a well-trained cross-try skier in whom the percentage of type I fibres decreased from 81% to 57% during eks of immobilization due to an injury (Jansson, Eriksson and Häggmark, personal munication 1978). Thus it might be assumed that not only specific training stimulus but inactivity might induce a conversion of type I to type II muscle fibres.

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TABLE I. Percentage of the different fibre types after aerobic and anaerobic training.

Subject	"Aerobic" training					Anaerobic training				
	I	II A	II B	II C	II B/II A	I	II A	II B	II C	II B/II A
1 GW	60	22	18	0	0.8	34	22	29	15	1.3
2. TB	83	15	1	1	0.1	77	7	4	12	0.6
3 CI	68	30	2	0	0.1	48	16	29	7	1.8
4 MH	66	14	20	1	1.4	48	8	11	14	0.4
Mean	69	20	10	1	0.6	52	18	18	12	1.0
SD	10	7	10	1	0.6	18	9	12	4	0.6

and denotes significant difference between the aerobic and anaerobic training at the level of p<0.0 and p < 0.01 respectively

for myofibrillar ATPase (Brooke and Kaiser 1970) to identify the fibre types. 300 (180-4 fibres were classified from each biopsy

After "anaerobic training, all subjects had a lower percentage type I and a higher pe centage type II C fibres than after aerobic" training. Three subjects also had a higher pe centage type II A + II B fibres and an increased II B/II A ratio after "anaerobic trai (Table I). Thus, it seems reasonable to assume that a conversion of type I to type II C fibr had occurred in subjects 1 and 2 due to "anaerobic" training and that a conversion in t opposite direction had occurred in subject 3 and 4 due to "aerobic" training. The type II fibres might be fibres in transformation from type I to type II A or in the reversed directi as hypothetized in Fig. 1 Support for this hypothesis might be found in the fact that ther a continuity in the stain intensity within the type II C fibre population (Fig. 2) from fib

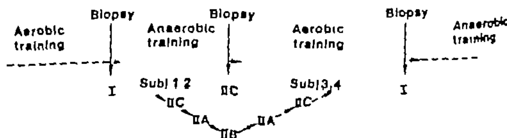


Fig. 1 A hypothetic model for fibre type transformation induced by training



Fig. 2. Serial sections from vastus lateralis (subj. 1 anaerobic training) stained for myofibrillar ATPase after preincubation at pH 10.3 (A), pH 4 6 (B) and pH 4.3 (C). Type II C fibres with stain intensity ... to type I fibres (II C') and to type II A fibres (II C") are present.

I. Age, weight, maximal oxygen uptake, muscle fiber area and fiber type distribution in untrained, cal education students and endurance-trained subjects. Values are the mean for each group.

ps	Number of fibers	Age (y.)	Weight (kg)	Maximal oxygen uptake (ml kg⁻¹ min⁻¹)	Muscle fiber area (μm)²	Mean fiber type distribution Type I	Type II A	B	Other
[illegible]ained [illegible]	1 649	25 (21 31)	76.4 (65.5–95 0)	50 8 (44 4–57 7)	4 266 (2 631–6 504)	42.4 (22.3–60.7)	33.2 (21 1 50 8)	18.7 (0 0–36.1)	5.7 (0 0–14 6)
[illegible]cal [illegible]ation [illegible]dents	889	23 (19–33)	74 4 (64 5–82.0)	62.6 (57.5–66.0)	4 105 (3 513–5 224)	50.9 (42.1 62.7)	31.6 (21.5–52.0)	11.9 (0 0–16.9)	5 6 (0.0–10.1)
[illegible]rance-[illegible]ined [illegible])	1 739	24 (18–34)	70.1 (58.7–75 5)	74.2 (58.7–79 9)	4 409 (3 685–6 854)	67.6 (43.9–84.1)	20 1 (10.9–42.5)	6.1 (0 0–17.1)	6 2 (0 0–9 2)

Number of subjects. ( ): Range.

Another factor which may contribute to the dispersion in individual CA values seen in 1 a and b is the relatively large individual differences in fiber type composition (Table I ge), since there is a clear difference in CA for the various fiber types (Andersen and Henson 1977 Ingjer 1978). These differences in CA caused by the large individual variations fiber area and fiber type composition become much smaller when the mean values for each oup is used (Fig. 1). This is caused by the relatively small differences in the percentage

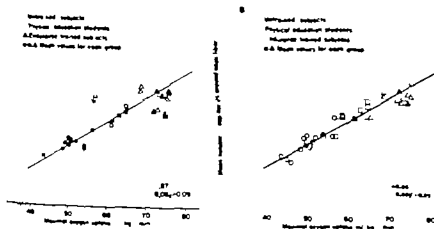


Fig. 1 a. Diagram showing relation between mean number of capillaries around each fiber and maximal oxygen uptake (ml kg⁻¹ min⁻¹). Each point represents values from one subject.

Fig. 1 b. The same relation as in Fig. 1 a, but the individual CA values are here corrected for differences in fiber area between the subjects.

Acta physiol scand, 1978 104 238–240

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Maximal aerobic power related to the capillary supply of the quadriceps femoris muscle in man

By

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The capillary supply of the quadriceps femoris muscle in man has been the subject of sev investigations recently (e.g. Andersen and Henriksson 1977 Brodal *et al* 1977 Ingjer a Brodal 1978) Separate untrained and endurance-trained groups (Andersen 1975, Brod *et al* 1977 Saltin *et al* 1977 Ingjer and Brodal 1978) or one group before and after trai (Andersen and Henriksson 1977) have been studied These studies show that increa maximal aerobic power is accompanied by increased capillary supply However sinc groups and the range in maximal oxygen uptake are small in these studies, they do elucidate the type of relationship between these parameters. This requires a large numbe subjects with a continuous variation in maximal oxygen uptake ranging from low to v high values. Accordingly the present investigation was undertaken to study the relat between capillary supply and maximal aerobic power in such a group of male subjects.

In two previous studies (Brodal *et al* 1977 Ingjer 1978) the mean values for the capi supply of the vastus lateralis of the quadriceps femoris muscle in untrained and endura trained groups have been given The individual values from untrained and endurance-tra subjects together with capillarization values from 9 physical education students with medi aerobic capacity in between the two other groups is presented in this study (Table I). methods used for taking the biopsies, and for determination of the maximal oxygen upt the capillarization values, the fiber areas and the fiber type composition, have been publis earlier (Brodal *et al* 1977 Ingjer 1977) and will not be described here.

Fig. 1 shows that there is an approximately linear relationship between the average num of capillaries around each fiber (CA) and the maximal oxygen uptake. There are no statist differences in fiber areas between the three groups (Table I) and when individual values examined, there is no relation between fiber area and maximal oxygen uptake. Howe individual variation in fiber area (Table I see range) may in extreme instances cause 20-2 difference in CA between subjects with identical maximal oxygen uptake, as calculated f the relationship between fiber area and CA given by Ingjer (1978). This fact explains par the dispersion of individual values seen in Fig. 1 a, as shown in Fig. 1 b where correc has been made for individual differences in muscle fiber area. A choosen muscle fiber of 4 400 μm and an increase on 0.56 capillaries around each fiber for each 1 000 μm incr in fiber area (Ingjer 1978) has been used.

I. Age, weight, maximal oxygen uptake, muscle fiber area and fiber type distribution in untrained, al education students and endurance-trained subjects. Values are the mean for each group

	Number of fibers	Age (y)	Weight (kg)	Maximal oxygen uptake (ml kg⁻¹ min⁻¹)	Muscle fiber area (μm)²	Mean fiber type distribution Type I	Type II A	Type II B	Other
...ned	1 349	25 (21 31)	76 4 (65.5–95.0)	50 8 (44.4–57 7)	4 266 (2 631–6 504)	42.4 (22.3–60.7)	33.2 (21 1 50 8)	18.7 (0 0–36.1)	5.7 (0 0–14 6)
...al ...tion ...ents	839	25 (19–33)	74.4 (64.5–82.0)	62.6 (57.5–66 0)	4 105 (3 513–5 224)	50 9 (4..1 42.7)	31.6 (21.5–52 0)	11.9 (0 0–16.9)	3.6 (0.0–10.1)
...rance-...ned)	1 739	24 (18–34)	70 1 (58.7 73.5)	74 2 (58.7–79.9)	4 409 (3 685–6 854)	67.6 (43.9–84.1)	20 1 (10.9–42.5)	6.1 (0.0–17.1)	6.2 (0 0–9.2)

Number of subjects. ( ): Range.

Another factor which may contribute to the dispersion in individual CA values seen in . 1 a and b is the relatively large individual differences in fiber type composition (Table I, ge), since there is a clear difference in CA for the various fiber types (Andersen and Henriksson 1977, Ingjer 1978). These differences in CA caused by the large individual variations fiber area and fiber type composition become much smaller when the mean values for each oup is used (Fig. 1). This is caused by the relatively small differences in the percentage

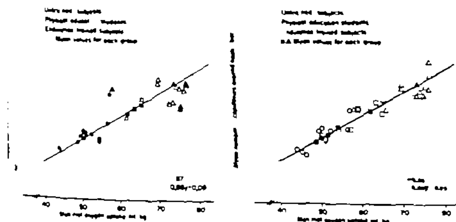


Fig. 1 a. Diagram showing relation between mean number of capillaries around each fiber and maximal oxygen uptake (ml kg⁻¹ min⁻¹). Each point represents values from one subject.

Fig. 1 b. The same relation as in Fig. 1 a, but the individual CA values are here corrected for differences in fiber area between the subjects.

number of type I + II A fibers between the various groups, and the fact that type I and II have both high capillary supply while only type II B has low capillary supply (Andersen & Andersen and Henriksson 1977 Ingjer 1978), and because a previous study (Ingjer 1979) shown that a 1 000 μm^2 increase in fiber area increases CA by approximately 0.56, the difference in mean fiber composition can only account for a 3–5 % increase in the CA from the untrained to the endurance trained group in this study and the difference in mean fiber area only for 1–3 % increase, while the real difference is about 46 %. This increase in capillary supply with increasing maximal oxygen uptake corresponds reasonably well with results on oxidative enzyme activities (e.g. Costill *et al* 1976, Bylund *et al* 1977), mitochondrial content (e.g. Gollnick *et al* 1971 Hoppeler *et al* 1973 Bylund *et al* 1977), and capillary supply from other studies (e.g. Andersen and Henriksson, 1977).

In conclusion, the present investigation suggests that the capillary supply of the vastus lateralis of the quadriceps femoris muscle in man increases linearly with increasing maximal oxygen uptake, and that the greater part of this increase can be accounted for by the effect of endurance training, and only to a very limited extent by differences in fiber type composition and fiber area between the groups. This points to the capillary supply as one important factor for the aerobic work power

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Anticholeretic effect of somatostatin in anesthetized dogs

By

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...tetradecapeptide somatostatin, originally found to be an inhibitor of growth–hormone–...sing factor has been demonstrated to be active in the gastrointestinal tract. It thus ...bits gastrin-induced gastric acid and secretin-induced pancreatic juice (Review Efendić ...l. 1978). An inhibiting effect on hepatic bile output would therefore be expected. The ...ent paper is a preliminary report on this subject, which appears not to have been studied ...iously

Material and methods

...study was performed in 5 mongrel dogs, weight 30–40 kg. Anesthesia was induced and ...tained with pentothal sodium and nitrous oxide in oxygen. The animals were endo...heally intubated and ventilated by an Engstrom respirator Arterial blood gases and ...-base status were controlled regularly and corrected when necessary Via a small sub...al incision, the cystic duct was ligated and common duct fistula created close to the ...hilus. The bile output was measured by weighing each three-minute fraction of bile ...an accuracy of 0.01 g. Intra-arterial blood pressure was measured by a catheter located ...e abdominal aorta, inserted via femoral artery and connected to a mechano-electrial ...sducer Statistical analyses were performed by the use of Student s t-test for independent ...es.

...omatostatin (Kabi) was administered as constant rate i.v infusions in the following ...ner I. Three infusions, dosage 0.2 $\mu g\ kg^{-1}\ min$ duration 9 min. II Four infusions, ...age 1 $\mu g\ kg^{-1}\ min$ duration 9 min. III Nine infusions, dosage 1.0 $\mu g\ kg^{-}\ min^{-1}$ ...ation 1 min.

Results

...e-minute infusions

...e bile output decreased following somatostatin. Maximum decrease varied interindi...ually between 40 and 95 per cent after the 0.2 μg dose, and between 45 and 85 percent ...r the 1.0 μg dose. Significant reduction of bile output occurred 6 min after onset of ...sion, and maximum effect was obtained 9–12 min after onset of infusion. The bile out... remained at a significantly depressed level during 12 min after termination of infusion. ...ere was no change in mean arterial blood pressure.

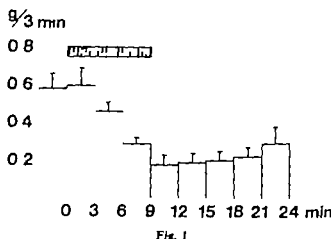


Fig. 1

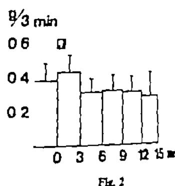


Fig. 2

Fig. 1 Bile output following i.v. infusion of somatostatin. 7 infusions, dosage 0.2-1.0 μg·kg⁻¹·min⁻¹ duration 9 min. Vertical lines indicate S.E.M. $p < 0.05$ $p < 0.01$

Fig. 2. Bile output following i.v. infusion of somatostatin. 9 infusions, dosage 1.0 μg·kg⁻¹·min⁻¹ duration 1 min. Vertical lines indicate S.E.M.

There was no statistically significant difference between low or high dosage in magnitude, onset or duration of the response. Bile output values for the two groups together are presented in Fig. 1.

One-minute infusions

The bile output did not change following one minute infusions of somatostatin, 1.0 μg kg⁻¹ min⁻¹. A slight decrease of bile output, observed in most experiments, was not statistically significant. Mean arterial pressure was unchanged. Bile output values are presented in Fig. 2.

Discussion

The present work demonstrates that somatostatin is an anticholeretic agent. The 0.2 μg dosage was chosen since it is known to inhibit gastric acid and pancreatic juice secretion. Obviously this dosage has also a maximum inhibiting action on hepatic bile output, equal to that obtained following the 1.0 μg dosage of equal duration. The anticholeretic effect seems to be more related to the duration of infusion than to the dosage administered. The 9-min infusions at the lower dosage, 0.2 μg kg⁻¹ min⁻¹, were more efficient than 1-min infusions at the higher dosage, 1.0 μg kg⁻¹ min⁻¹. The minor effect of brief infusions appears to be due to a rapid inactivation of the substance. A calculation based on the duration of the effects, suggests that somatostatin has a half life time of between 2 and 3 min under the present circumstances. This value is in accordance with earlier findings in man (Efendić, unpublished data).

The anticholeretic effect of somatostatin shows a typical slow pattern. This is in contrast to all other effects of the substance, which are immediate. It is also different from the effect of Substance P, which is the only physiological substance previously known to inhibit bile output. Substance P is believed to exert a direct inhibitory action on sodium depen-

cular bile output (Holm *et al.* 1978). The delayed effect of somatostatin suggests that an indirect action, inhibiting gut hormone-induced bile secretion. This mechanism e further studied.

Summary

...ostatin, 0.2–1.0 μg kg^{-1} min^{-1} was administered i.v. in 5 anesthetized dogs. Following infusions, hepatic bile output was found to decrease by approximately 50% after a ...y of 6 min. The mode of action suggests that somatostatin inhibits hormone-induced ...utput.

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Acta physiol. scand. 1978, 104, 244-246

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The effect of food ingestion on circulating neurotensin-like immunoreactivity (NTLI) in the human

By

M. L. MASHFORD, GÖRAN NILSSON, ÅKE RÖKAEUS and SUNE ROSELL

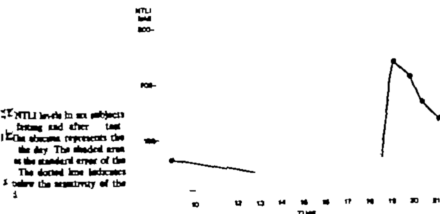
Neurotensin is a tridecapeptide isolated from the bovine hypothalamus and the s... bowel (Carraway and Leeman 1973, 1976). Immunohistochemical studies have shown th... the gut contains glandular cells reacting with antibodies to neurotensin (Orci *et al.* 19... Sundler *et al.* 1977, Helmstaedter *et al.* 1977). In anesthetized dogs higher concentrations... NTLI have been found in blood draining from the small intestine than in the arterial blo... (Rosell *et al.* 1978). Thus the experimental data obtained so far suggest that neurot... may be a hormone which is stored in and released from the small intestine. One requi... ment for a circulating material to be regarded as a hormone is that its concentration i... blood varies in a reproducible way in relation to some physiologically important ev... This study reports the effects of fasting and food intake and also of exercise on periphe... venous concentrations of NTLI in humans. During fasting water was taken *ad libitum*. T... subjects were 6 healthy males aged from 23 to 47 years.

Blood was collected into chilled heparinized tubes and spun at 4°C. The plasma ... stored at −20 C until subjected to radioimmunoassay for NTLI. The antibody (9-1... used in the assay was raised in rabbits. Details about the method will be reported elsewh...

The antibody reacts with neurotensin (NT), NT (1–12), (Gln⁴) NT and (Glu⁴) NT (1-... but not with NT (4–13) or smaller C-terminal fragments.

It shows effectively no cross-reactivity with VIP, secretin, cholecystokinin-33, cholec... kinin-39, pancreatic glucagon, substance P, insulin, somatostatin, GIP, gastrin-17, gastri... or with trypsinized gastrin-34 which presumably contains the N-terminal heptadecapep... The assay can measure levels down to 10 pM.

The mean concentration of NTLI in 6 subjects after an overnight fast was 65 ± 12 ... (M ± S.E.) (Fig. 1). Continued fasting was associated with further fall in blood conce... tion which for 3 of the subjects resulted in concentrations below the sensitivity of the a... At 1830 h a test meal consisting of an apéritif of 40 ml whisky, white bread roll and 4... butter, steak, fried potatoes and béarnaise sauce, 100 ml wine and coffee was commenced... All post prandial concentrations of NTLI were significantly elevated. The mean concentra... tion at 45 min was 233 ± 86 pM. The NTLI concentration fell thereafter but remained... elevated in all subjects for the 2 h and 45 min during which sampling continued. In anothe... experiment, two of the subjects fasted overnight. Blood samples were taken during the... morning and at 1300 h 40 ml whisky were consumed and blood ... continued at 1...



...NTLI levels in six subjects fasting and after ... test. The abscissa represents the ... the day. The shaded area ... the standard error of the ... The dotted line indicates ... below the sensitivity of the ...

...ntervals for 1 h. No significant change in blood concentration of NTLI was seen but ...a light snack consisting of a sandwich and 100 ml youghurt taken at 1500 h, a pro-...ced increase of NTLI occurred. Two subjects fasted overnight and drank 100 ml black ...e at 1200 h. There was no response of NTLI to coffee. Three of the subjects in varying ...s of physical training ran for 40 min at close to each individual's maximal capacity ...dged by the pulse rate. There was no consistent change in the blood concentration of ...I.

...he data indicate that the ingestion of food appears to exert an overriding influence ...he blood concentrations of NTLI. The meals consumed contained fat, proteins, carbo-...rates and the influence of distension of the esophagus and the stomach may also be of ...ortance. Alcohol and coffee which were also consumed, did not seem to greatly influence ...level when taken alone.

...he NTLI cannot be termed neurotensin since it has not been isolated and chemically ...racterized. Moreover experimental data indicate that some of the actions of neurotensin ...y be caused by substance(s) formed from neurotensin (Rosell *et al.* 1978). Since the anti-...dy is directed towards the N-terminus of the neurotensin molecule shorter N-terminal ...quences of neurotensin may have been detected. The consistent pattern of change in the ...ood concentration of NTLI associated with food intake suggests that the circulating ...ptide or peptides are subserving some hormonal function in relation to eating but the ...ture of such regulatory function is not known. The most sensitiv target organs for neuro-...nsin so far are the upper gastrointestinal tract and the adipose tissue. Thus, i.v. infusion ...f neurotensin in anesthetized dogs (5–30 pmol kg^{-1} min^{-1}) inhibits motor activity in the ...ntral part of the stomach (Andersson *et al.* 1977), inhibits gastric acid secretion (Andersson ...*et al.* 1976), increases the blood flow in the gastrointestinal tract and has a vasoconstrictor ...ction in subcutaneous adipose tissue but does not appreciably change blood pressure ...Rosell *et al.* 1976). At higher doses the blood glucose concentration is elevated (Carraway ...Demers and Leeman 1973, Rosell *et al.* 1976).

This investigation has been supported by the Swedish Medical Research Council (3518) and by Åke W...rgs Stiftelse. We thank Dr R. Carraway (Harvard Univ. Mass.), Dr K. Folkers (Univ. of Texas, Austin, ...), Dr V. Mutt (Karolinska Institutet) for generous supply of various peptides.

Addendum

During preparation of this manuscript Besterman *et al.* (The Lancet, pp. 785-78, [illegible] reported that a test-meal increases the concentration of neurotensin in blood.

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Failure of buccal stimulation with NaCl to evoke an homeostatic response related to body sodium

By

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-pharyngeal receptors are believed to be involved in the control of water and salt intake of water excretion (Epstein *et al.* 1973). Specifically Nicolaidis (1969) working with ...ated, anesthetized rats, has shown that buccal stimulation with tap water increased ... flow within 1 min whereas buccal stimulation with 5% NaCl solution decreased urine ...thin 1 min. We deemed it pertinent to determine whether or not oro-pharyngeal ...ptors were also involved in the control of Na excretion as are some post-pharyngeal ...ptors (Daly *et al.* 1967, Carey *et al.* 1976).

...dult Sprague-Dawley rats were anesthetized with Inactin (Promonta) 120 mg/kg i.p. ...nimals) or with amobarbital 150 mg/kg i.p. (2 animals) with additional small doses. ...ureters, one femoral vein and one femoral artery were cannulated for urine sampling, ...infusion of saline (0.85%) or 5% mannitol on saline, and the recording of blood pressure. ...h the rat on its abdomen the tongue was fixed in a plastic cup. From a tube the NaCl ...tion (20 drops/min) ran over the tongue into the the buccal cavity and out of the ...th. In 3 expts. the left kidney was denervated by pedicle stripping and application of ...nol crystals and in one of these animals vasopressin was infused for the last half of the ...t. Experimental observations were made in hydropenia (no femoral vein infusion) and ...owing volume expansion (3 to 5 ml of 0.85% NaCl i.v. followed by the same or 5% ...nitol in saline at 4 to 6 ml/h). Animals were kept at about 37°C body temperature by a ...ro-controlled heating pad.

The results on Inactin anesthetized animals were as follows. In one hydropenic animal ...th both kidneys intact, 1 M NaCl on the tongue (4 trials) caused complete cessation of ...ine flow during or immediately following the application. In an additional hydropenic ...imal with one kidney denervated, dripping 0.5 M NaCl onto the tongue for 15 min (3 ...als) appeared to decrease Na excretion during and afterward in both innervated and ...nervated kidneys (Fig. 1 lower left). After volume expansion in two other rats 0.5 M (2 ...ials) and 1 M NaCl (2 trials) had little effect on the innervated side and may have caused ...slight reduction in Na excretion on the denervated side (Fig. 1 upper left). During vaso-...ressin (ADH) infusion, 1 M NaCl on the tongue (1 trial) appeared to be without effect.

In the amobarbital anesthetized animals the results were quite different. Urine flow and ...Na excretion were much greater and 0.5 M NaCl and 1 M glucose were without effect on

Addendum

During preparation of this manuscript Besterman *et al.* (The Lancet, pp. 785-788, [illegible]) reported that a test-meal increases the concentration of neurotensin in blood.

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Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men

By

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Abstract

ÖRLANDER, J., K. H. KIESSLING, L. LARSSON, J. KARLSSON and A. ANIANSSON. *Skeletal muscle metabolism and ultrastructure in relation to age in sedentary men*. Acta physiol. scand. 1978. *104* 249–261

In order to find out if there are age-related changes in human skeletal muscle metabolism or ultrastructure, biopsy material from 56 sedentary men aged 22–65 years was studied by means of enzyme activity determinations, histochemistry and quantitative electron microscopy. For comparison, a younger (16–18 years) and an older (66–76 years) group were included. These subjects were relatively more active. There was an increase in percentage of slow twitch fibres with age. Mitochondrial volume fraction decreased with age, mainly due to diminished mean mitochondrial volume. In spite of this, no overall decrease in the activity of five enzymes, representative of the major pathways in energy metabolism, was observed. Thus, increased amounts of enzymes per unit mitochondrial volume are implicated. Lipofuscin was more frequently found in the older groups. Correlations were present between fibre type distribution and oxidative enzymes, as well as between different enzymes. It was concluded, that the decrease in maximal oxygen uptake and muscular strength in aging humans probably may not be explained in terms of deteriorating skeletal muscle energy metabolism.

Key words: aging, muscle metabolism, muscle ultrastructure

It is a well-known fact that the physical performance capacity will decline during middle-age and senescence. The maximal oxygen uptake capacity, the common measure of fitness, will decrease (Åstrand 1960), as well as the muscular strength (Ufland 1933, Fisher and Birren 1947, Asmussen and Heebøll-Nielsen 1961). It is still unsettled to what extent these effects are due to biological aging or to decreasing physical activity during old age. Training may counteract the physical decline (e.g. Grimby and Saltin 1966), although not completely, indicating that both decreasing physical activity and biological aging are involved.

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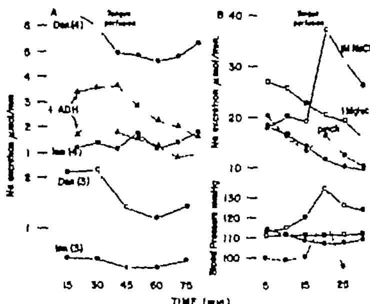


Fig. 1 Effect of 0.3 and 1 M ... 1 M glucose, and tail pinch ... excretion in fasting (left) ... barbital (right) anesthetized

blood pressure or Na excretion (Fig. 1 right side) However 1 M NaCl on the ... initiated chewing and tongue withdrawal movements, a rise in blood pressure, and a ... increase in Na excretion Pinching the tail (5 min) evoked an approximately compar... rise in blood pressure but a lesser increase in Na excretion. A second animal gave ... results.

The results are believed to show that whereas 1 M NaCl may elicit either a reduction ... urine flow and Na excretion or an increase, depending on experimental conditions, ... neither of these responses may be considered as specific homeostatic responses used to ... cipate changes in body sodium. The cessation of urine flow and occasional reduction ... excretion were presumably the result of release of endogenous vasopressin whereas t... increase in Na excretion seen in the amobarbital anesthetized animal was probably re... to the arousal engendered by the hypertonic salt tail pinch seemed to have a similar ef...

In conclusion, these studies provide no evidence for oro-pharyngeal receptors for Na ... are involved in controlling Na excretion by the kidney

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Table I. Anthropometrical characteristics of the subject groups. Values are means ± S.E., and ranges are given within brackets. * and ** denote $p<0.05$ and $p<0.01$, respectively, when compared with the reference group (20–29).

Group	Number of subjects	Age (years)	Height (cm)	Weight (kg)
18	6	17±0 (16–18)	179±2 (174–186)	70.6±3.4 (61.2–79.0)
29	11	26±1 (22–29)	182±2 (170–190)	76.2±3.2 (58.1–95.0)
39	16	34±1 (30–39)	181±1 (172–194)	82.8±3.0 (64.7–108.7)
49	11	43±1 (41–48)	182±2 (174–190)	80.5±4.2 (62.4–111.5)
59	9	54±1 (51–57)	180±2 (171–187)	81.6±2.5 (74.5–98.8)
65	9	61±1 (60–65)	175±1 (169–180)	74.4±3.6 (61.8–98.7)
76	7	70±1 (66–76)	172±2* (164–180)	77.0±2.3 (67.0–87.0)

3-hydroxyacyl-CoA dehydrogenase, HAD (E.C.1.1.1.35), the citric acid cycle by citrate synthase, CS (E.C.4.1.3.7), and the respiratory chain by cytochrome oxidase, cytox (E.C.1.9.3.1). Homogenates were prepared and assays performed as described previously (Örlander et al. 1977). PFK activity was determined by the method of Shonk and Boyer (1964), LDH and HAD according to Bass et al. (1969), CS as described by Srere (1969) and cytox according to Wharton et al. (1969). Protein was estimated by the method of Lowry et al. (1951).

Electron microscopy. Small pieces of muscle tissue were processed for electron microscopy and micrographs were taken for morphological analysis according to Weibel (1969). Details of the procedure have been described before (Örlander et al. 1977). Since the size distribution and average shape of skeletal muscle mitochondria are not known, the computation of mitochondrial number and mean volume involves approximations (Weibel 1969, Örlander et al. 1977). This means that these values are only rough estimates in the true ones. Because the electron microscopical technique is extremely time-consuming, this part of the investigation was limited to 48 subjects.

Statistics. Linear regressions and correlation coefficients were calculated by standard procedures. The significance of correlation coefficients was determined with t-test. To test differences between group means, Student's t-test was applied, $p<0.05$ being considered significant.

Results

In Fig. 1–4, group mean values are given at the respective group mean ages, along with regression lines, based on individual values. Regression lines were calculated from values of the 20–65 year groups only. Where no significant correlation was obtained, the best fitting regression line was still indicated (broken line).

Fibre type distribution. The mean percentages of ST fibres in the various groups are given in Fig. 1. A significant ($r=0.46$, $p<0.001$) linear increase in % ST fibres with age was observed in the 20–65 year groups, and also when the 66–76 year group was included ($r=0.44$, $p<0.001$). No significant differences in fibre type distribution within the FT fibre population were observed (Table II), except for the total lack of c fibres in the oldest group

There is a close connection between the physical performance capacity and the aerobic capacity of the working muscles. As has been shown extensively by numerous investigators during recent years, a change in the level of physical activity will produce corresponding metabolic alterations in the muscles involved (for a review, see Holloszy and Booth ...). With this in mind, the possibility that an age-related deterioration of the muscle metabolism might be a cause for the decreasing working capacity during aging suggests itself.

Rather few reports have appeared on metabolic changes in skeletal muscle during age. Furthermore, most investigators have studied the laboratory rat (Syrovy and Gutmann 1970, Rockstein and Brandt 1961, Ermini and Verzár 1969, Ermini *et al.* 1971, Ermini and Szelényi 1972, Schmukler and Barrows 1966, Bass *et al.* 1975). In certain respects the results of these studies are conflicting, and they give no clear picture of the metabolic ... during senescence.

The objective of the present investigation has been to survey some parameters indicative of the state of the energy metabolism in human skeletal muscle in relation to age. In order to achieve this, muscle biopsy material from subjects aged 16–76 years has been examined enzymatically, histochemically and electron microscopically.

Material and methods

Subjects. A total of 69 healthy men volunteered to participate in the investigation, each having given informed consent. The major part, 56 men aged 2...–65, were recruited from the staff of an ... company. They were all white-collar workers, engaged in no or light physical activity during their spare time, and were thus considered sedentary. 24 of them were identical with the subjects of a previous study (Örlander *et al.* 1977). Data from muscle strength measurements on this subject population are presented separately (Larsson *et al.* 1978 b). Briefly, they show a decrease in isometric as well as dynamic strength in the oldest groups.

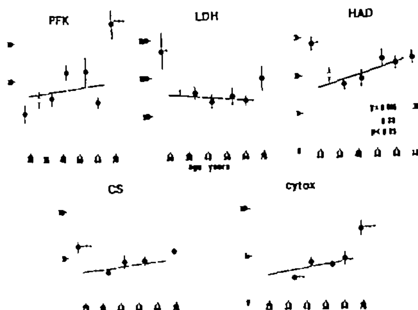
The seven oldest men, 66 to 76 years old, were pensioners with various previous occupations and comparatively active for their age. The remaining subjects were 6 high school students aged 16–18. All but ... of these were physically active and participated in various sports. Strength measurements in the ... (unpublished) revealed that these subjects were significantly stronger than sedentary men of the same age.

A division into 7 age groups was made with each decade making up one group, except for the youngest and the two oldest groups. The rationale for the deviations from the decade division was that the youngest (16–18) and oldest (66–76) groups were distinct from the others, being non-sedentary, and were best treated separately, whereas the 60–65 year group resulted from 65 being the age of retirement from the ... company. The 20–29 year group, comprising the youngest adult subjects, was chosen as reference group. Some anthropometrical characteristics of the subject groups are given in Table I.

Muscle biopsies. Muscle tissue was obtained from the *vastus lateralis* muscle with a needle technique (Bergström 1962). All biopsies were taken at 8–10 a.m. with no preceding exercise. Biopsy material was obtained for histochemistry, for determination of enzyme activities and for electron microscopy.

Histochemistry. A muscle piece was trimmed, oriented, mounted in histoclad, frozen in isopentane cooled by liquid nitrogen and stored at −80°C until analyzed. Serial transverse sections (10 µm) were cut in a cryostat at −20 C, mounted and stained for myofibrillar ATPase (Padykula and Herman 1955, Guth and Samaha 1969). Micrographs of the stained sections were taken and fibre classification into slow twitch (ST, type I) or fast twitch (FT, type II) (Engel 1962) was made in 62 subjects. In 44 subjects, a further study was made in order to distinguish the a, b and ... subgroups of FT fibres (Brooke and Kaiser 1970, Dubowitz and Brooke 1973). For technical reasons, no histochemical study could be made in the youngest group.

Enzyme assays. To get a picture of the state of the energy metabolism, five enzymes were chosen to represent these for the major pathways. Glycolysis was represented by phosphofructokinase, PFK (E.C.2.7.1.11), lactate fermentation by lactate dehydrogenase, LDH (E.C.1.1.1.27), fatty acid β-oxidation



2. Enzyme activities in vastus lateralis in the various age groups. Activities are given as μmol min^{-1} (wet wt)$^{-1}$ for phosphofructokinase (PFK), lactate dehydrogenase (LDH), 3-hydroxyacyl-CoA dehydrogenase (HAD) and citrate synthase (CS), and as μmol O_2 min^{-1} (g wet wt)$^{-1}$ for cytochrome oxidase (cytox). For further explanations, see legend to Fig. 1. Numbers of subjects (from left to right) 6, 11 16, 9 9 and 7. Broken lines indicate non-significant regressions. and denote p 0.05 p < 0.01 and p 0.001, respectively when compared with the reference group (20–29)

Cytox was lowered in the 30–39 and elevated in the 66–76 year group (p 0.01 and p 0.001 respectively). No correlation with age was present in the 20–65 year groups. No significant inter-group differences in muscle protein concentration were observed.

When the data from all subjects, regardless of age, were treated together a considerable number of significant correlations between different parameters were observed. The activities of HAD (r 0.34 p 0.01), CS (r 0.36, p 0.01) and cytox (0.39 p 0.01) were positively correlated with the percentage of ST fibres (data from 62 subjects). Correlations between enzyme activities, indicating relationships between metabolic pathways, are listed in Table III. The strongest correlations were found between oxidative enzymes. Significant correlations were absent in the pairs PFK LDH, PFK HAD and LDH–cytox.

TABLE III. Correlations between enzyme activities in the vastus lateralis. Data from all subjects (n 69) are included in the calculations. PFK phosphofructokinase, LDH lactate dehydrogenase, HAD 3-hydroxyacyl-CoA dehydrogenase, CS citrate synthase, cytox cytochrome oxidase, correlation coefficient, not significant.

	Enzyme pair									
	PFK LDH	PFK HAD	PFK CS	PFK cytox	LDH HAD	LDH CS	LDH cytox	HAD CS	HAD cytox	CS cytox
	0.16	0.04	0.26	0.34	0.27	0.26	0.14	0.73	0.45	0.62
p			0.05	0.01	0.05	0.05		0.001	0.001	0.001

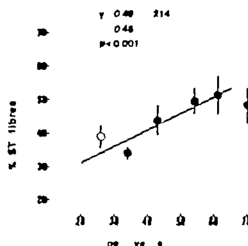


Fig. 1. Percentage slow twitch (ST) fibres in *m. vastus lateralis* in the 20–76 year groups. Mean ± SE for each age group are indicated at the respective mean ages (cf. Table 1). The number of subjects in each group was (from left to right) 11, 16, 11, 8, 9 [illegible]. The regression line was calculated from the individual values of the 20–65 year groups. Unfilled [illegible] reference group (20–29).

The histochemical data pertaining to the 20–65 year groups are presented in greater detail separately (Larsson *et al.* 1978 a). Here, they have been included as a background for the metabolic data.

Enzyme activities. Mean activities of the five selected enzymes in the [illegible] muscles of the various groups are shown in Fig. 2. With the exception of HAD no significant correlations with age were found in the 20–65 year groups.

The PFK activity was significantly higher in the 40–49 and 66–76 year groups than in the reference group ($p < 0.05$ and $p < 0.001$, respectively). For the 20–65 year groups, there was no correlation with age, but when the 16–18 and 66–76 year groups were included, a significant correlation ($r = 0.44$, $p < 0.001$) was obtained.

LDH showed no age differences in the 20–65 year groups. The value of the 16–18 year group was significantly elevated ($p < 0.05$).

For HAD there was a significant ($r = 0.28$, $p < 0.05$) positive linear relationship with age in the 20–65 year groups. This held true also when the 66–76 year group was included ($r = 0.31$, $p < 0.05$). The 16–18 year group's value was significantly elevated ($p < 0.05$) when compared with the reference group.

No significant age differences in CS activity were observed in the 20–65 year groups. The values of the 16–18 and 66–76 year groups were elevated (both $p < 0.001$).

TABLE II. Distribution of fibre types within the fast twitch (type II) fibre population in the 20–76 year groups. Values are means ± S.E. * denotes $p < 0.05$ when compared with the reference group (20–29).

Age group	Number of subjects	% of fast twitch fibres		
		Subgroup a	Subgroup b	Subgroup c
20–29	6	57±3	34±4	9±3
30–39	11	61±4	34±3	5±2
40–49	7	70±7	27±7	3±1
50–59	7	61±7	36±7	3±1
60–65	6	61±8	32±9	7±2
66–76	7	52±6	48±6	0

IV Volume fractions of lipid droplets (means ± S E.) and the occurrence of lipofuscin in the various age groups. S Z. subsarcolemma zones

group	Number of subjects	Lipid droplets of cell volume	Lipofuscin-containing S Z. of total number of S Z.
8	6	0 47 ± 0.16	0 (10)[a]
9	5	0 61 ± 0 15	10 (10)
9	14	0 60 _ 0 10	5 (38)
9	4	1.09 ± 0.42	20 (10)
9	6	0 92 ± 0.18	30 (23)
55	6	0 87 + 0 32	30 (23)
76	6	0 46 ± 0 16	32 (19)

umber of examined S.Z. in each group ithin brackets.

d a significant (r = 0.36, p 0.05) negative correlation when the youngest and oldest oups ere included. No significant age differences in number of mitochondria per μm^3 sarcomere were observed

Fig. 4 gives the corresponding data for the subsarcolemma region. There was a linear crease with age in mitochondrial volume fraction (r 0 48, p < 0 01) in the 20–65 ar groups. This held true when the values of the 16–18 and 66–76 year groups were included (r 0 63 p 0 001). The alues of the three oldest groups were all significantly er (p 0 01) than that of the reference group. A linear decrease (= −0 37 p < 0 05) as observed also for the apparent number of mitochondria per unit volume in the 20–65 ar groups. The other groups did, however not conform to this relationship. No significant ge changes in apparent mean mitochondrial olume were present in the 20–65 year groups, ut by including the youngest and oldest subjects a negative correlation with age (r = 0.35 0 05) was obtained

The volume fraction of lipid droplets in the arious age groups is shown in Table IV gether with the incidence of observations of lipofuscin (ge pigment). There were no ignificant age differences in lipid droplet content, but some subjects in the 40–65 year groups showed unusually high alues. The occurrence of lipofuscin pigment granules, hich ere localized exclusively in the subsarcolemma region, increased up to 50 years and was then constant. Pigment was most frequently present in f bres with broad Z-lines, presumably ST fibres (Wroblewski and Jansson 1975). Of all observed pigment granules, 77 ere found in fibres with broad Z-lines and 15% in fibres with narrow Z-lines, whereas 8 could not be classified. W emphasize, however that the observations on lipofuscin are a by-product of the present investigation, and the alues should be regarded as rough estimates A systematic study of the subsarcolemma zone is needed for a reliable quantification of lipofuscin occurrence.

When all studied subjects (43) were treated together a significant correlation was found between fibrillar space and subsarcolemma space mitochondrial volume fractions (r 0 44 p 0 01) No correlations between fibre type distribution and mitochondrial structural data or between mitochondrial structural data and the activities of mitochondrial enzymes ere observed.

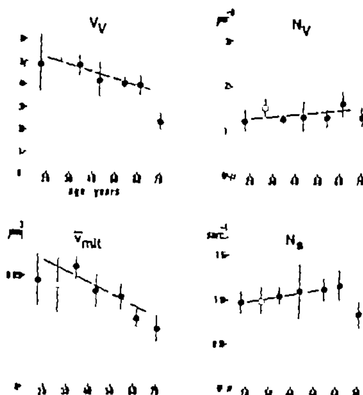


Fig. 3. Mitochondrial volume fraction (V_V), apparent number (N_V) and mean volume ($\bar{v}_{mit}$) ... number of mitochondria per sarcomere (N_s) in the fibrillar space of skeletal muscle fibres in the various age groups. Number of subjects (from left to right) ... 14, 4, 6, 6 and 7. For further explanations, see legends to Figs. 1 and 2.

Muscle ultrastructure. All micrographs showed normal gross ultrastructure as judged from visual inspection. The observations on mitochondrial volume fraction, mean volume and number of mitochondria per unit volume or sarcomere in the fibrillar region of muscle fibres are illustrated in Fig. 3.

The volume fraction of interfibrillar mitochondria tended to decrease with age ($p<0.1$) in the 20–65 year groups. When the significantly decreased ($p<0.001$) value of the 66- year group and the value of the youngest group were included, a significant correlation ($r=-0.44$, $p<0.01$) was obtained. The apparent mean mitochondrial volume showed the same pattern, a tendency ($p<0.1$) towards an age related decrease in the 20–65 year groups

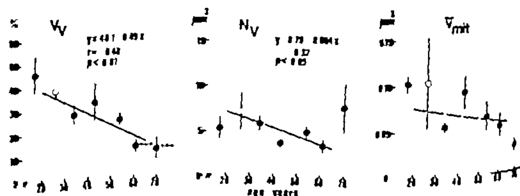


Fig. 4. Volume fraction (V_V), apparent number (N_V) and mean volume ($\bar{v}_{mit}$) of mitochondria in the sub-sarcolemma space of skeletal muscle fibres in the various age groups. Numbers of subjects (from left to right) 3, 4, 14, 4, 6, 6 and 6. For further explanations, see legends to Fig. 1.

younger groups (data not shown), thus supporting this suggestion. The trend towards r PFK activities with increasing age observed in the present study (Fig. 2) is in line the findings of Eriksson *et al.* (1973), who found much lower PFK activities in the levels of 11 13 year old boys as compared to the adult subjects studied by Gollnick (1972) Similarly Ritz and Kirk (1967) observed an age-related increase in PFK ity in human arteries.

increase in HAD activity with age (Fig. 2), and the similar but weaker trends for nd cytox are easily explained by the parallel increase in ST fibres (Fig. 1), since there positive correlations between the ST and these enzymes.

was considered of interest to subject the enzyme activity values to a correlation analysis rder to examine the relationships between metabolic pathways. The observed correla (Table III) support the validity of the concept of constant proportions between enzymes different metabolic pathways in muscle (Bass *et al.* 1969). The coordination of mitochondrial oxidative pathways is apparently strong, especially between fatty acid β-oxida- (HAD) and the citric acid cycle (CS) (*cf* Staudte and Pette 1972). Our results differ what from those obtained by Bylund *et al.* (1976) in human muscle, as they did not erve any correlations in the pairs PFK–CS, PFK–cytox, LDH–HAD or LDH–cytox, found one between PFK and LDH It is impossible at present to give an obvious explana- for these discrepancies.

A striking finding in the electron microscopic investigation was that the 16–18 and 66–76 r groups, being different from the others as to relative physical activity level and enzyme ivities (Fig. 2), fitted quite well in most trends seen in the 20–65 year groups (Fig. 3 4). is might indicate that there is an endogenous decline in total mitochondrial volume with e, independent—within limits—of the level of physical activity To maintain the meta lic capacity necessary during sedentary life, or to elevate it in response to physical activity mands during old age, an increase in oxidative capacity per unit mitochondrial volume en seems to be required. This might be achieved by an increase in the density of respira ry assemblies on the inner mitochondrial membranes (*cf* Kiessling *et al.* 1974 1975). contrast, training in younger persons causes the mitochondrial volume fraction to increase parallel with the oxidative capacity (Örlander *et al.* 1977)

It is further notable that the change towards decreased mitochondrial volume fraction th age proceeded along with an increase in the percentage of mitochondria-rich ST fibres ig 1). This means that on an equal fibre distribution basis, the decline in mitochondrial olume would be even more obvious

The data in Fig. 3 indicate that the above discussed decrease in mitochondrial volume fraction is caused primarily by a diminished mitochondrial mean volume in the fibrillar pace, the number of mitochondria being essentially constant The pattern is not quite as lear in the subsarcolemma space (Fig. 4), but if all age groups are included, the same onclusion is reached here. This is partly in accordance with the findings of Kiessling *et al.* 1973), who in a study of two age groups (mean ages 22 and 57) found an age-related decrease in mean olume with a simultaneous rise in number of mitochondria.

There was a more marked reduction with age in total volume of subsarcolemmal than of interfibrillar mitochondria (Fig. 3 4). It has recently been shown, in cardiac muscle, that

Discussion

The major finding of the present investigation was that no overall decrease in enzyme capacities of skeletal muscle energy metabolism occurs up to 65 years of age in healthy sedentary men Mitochondrial enzyme activities can actually be maintained or even elevated in spite of decreasing mitochondrial volumes

The question arises, whether systematic differences between the subjects of the different age groups might affect the present results. A hypothetical decline in oxidative enzyme activities (such a decline has been suggested for other organs, see Wilson 1973) could be obscured by a higher physical activity level or a greater percentage of highly oxidative muscle fibres in the older as compared to the younger subjects. Except for the youngest and oldest groups, major differences in physical activity level can be ruled out, since all subjects were more or less sedentary As to fibre type distribution, there was in the present material a significant increase in the percentage of ST fibres with age (Fig. 1). ST fibres have a greater oxidative potential than FT fibres (e g Essén et al 1975), and in the present study positive correlations were observed between the percentage of ST fibres and the activities of the oxidative enzymes (see Results) Calculations based upon the respective regression equations revealed that compensation for the greater proportion of ST fibres in the older groups tended to bring the group mean activities for these enzymes closer to those of the reference group Thus, there is little reason to assume that differences in fibre composition obscure any decrease in enzyme activities.

Possible causes for the observed change towards more ST fibres with age (Fig. 1) have been discussed elsewhere (Larsson et al 1978 a) Some additional comments will be made here

Fibre type distribution data from studies on untrained subjects by Gollnick et al (1972), Kiessling et al (1974) and Saltin et al (1976) are in good agreement with the present data. There is, on the other hand, an obvious dissimilarity with the study by Hedberg and Jansson (1976) on a representative sample of 16-year-old boys (mean 54 % ST fibres). The fact that all our groups had lower values might possibly be related to the observation of Hedberg and Jansson that the boys with a low percentage ST fibres were less active and less interested in physical activity than those with a high percentage It cannot be completely ruled out that the increase in ST fibres seen in the present study might reflect a sample bias towards interest in physical activity in the older groups

Generally the enzyme activity patterns of the 16–18 and 66–76 year groups deviated from those of the other groups (Fig. 1) The enzymatic profile of the youngest indicated greater anaerobic and fatty acid combustion capacities, no doubt due to their higher physical activity level, whereas the oldest subjects seemed better equipped for aerobic glycolysis when compared with the other groups The latter finding is hard to explain These old men were active for their age, participating regularly in activities such as bowling, but when compared with the younger groups there was no great difference. The observed high activities in the aerobic pathway for carbohydrate breakdown may be in some way necessary to maintain the capability of physical activity at this age Preliminary results from a study in progress of 7 less active men (mean age 71) show PFK CS and cytox activities similar to those of

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[illegible]el's (1963) "error catastrophe" theory of aging states that the accuracy of the cell's [illegible] synthesizing machinery will deteriorate with age, and the production of more and [illegible] error-laden enzyme molecules will eventually cause the death of the cell. The present [illegible]s, concerning the postmitotic skeletal muscle cell, further add to the body of data [illegible]ing no or small changes in enzyme activities during aging in a wide variety of organs [illegible]species (Finch 1972, Wilson 1973). Thus, the proposed "error catastrophe" does not [illegible] to markedly affect the amount of active enzyme molecules. There is, however, evidence [illegible] there is an increased production of inactive enzyme molecules during aging (Rothstein [illegible]). This is true for mouse muscle aldolase (Gershon and Gershon 1973). Errors in protein [illegible]esis might thus adversely affect the metabolism of the aging muscle, even though this [illegible]t readily apparent from enzyme activity values.

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In conclusion, the results of the present investigation strongly suggest that the decrea... physical performance capacity and muscular strength observed in humans during aging ... not be explained in terms of a deteriorating skeletal muscle energy metabolism. As judg... from the activities of representative enzymes from the major pathways (Fig. 2), the mus... cells of a 65–70-year-old man are still capable of maintaining as great a metabolic flow a... those of a young man. It therefore seems improbable, that the decrease in muscular streng... should be caused by a diminished supply of ATP (it can, however not be completely rule... out that there might be an impairment of the coupling between electron transport an... phosphorylation during old age) The answer might lie in an impaired function of the ... contractile apparatus itself the excitation–contraction coupling, or the neural influence... Turning to the age-related decrease in maximal aerobic power the present results spea... against a decreasing muscular oxygen utilization capacity as being a primary cause. Thus an impairment of the oxygen transporting system is implicated. As argued by Åstrand and Rodahl (1970) this may involve central circulatory (decreasing maximal heart rate, reduced stroke volume) and peripheral (capillary supply?) factors, as well as the efficiency of the regulation of circulation. In a recent longitudinal study Ka... Ekb...

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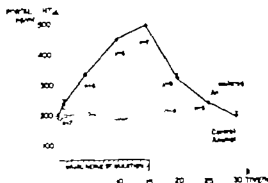


Diagram illustrating portal 5-HT ... cats immediately before, during ... er efferent electrical stimulation of ... cal vagi (mean values ± S.E.). ... k indicates that the level after 5 ... f stimulation is significantly in... compared ... th the baseline level ... 01 Student' ... -test). Dotted line ... ws the portal 5-HT in ... control ... shamoperated but not electrically ... ted.

... erent electrical stimulation of the cervical vagi is known to decrease the tissue 5-HT ... ntration in the duodenum, as shown by fluorometric assays (Hobenleitner *et al.* 1971). ... ncomitant degranulation of duodenal EC has also been demonstrated by the argentaffin ... ion (Tansy *et al.* 1971). By use of cytofluorimetry a vagally induced reduction of the ... cellular levels of 5-HT in EC has been confirmed in the cat (Ahlman 1976). All these ... ngs strongly suggest a vagal release of 5-HT from EC.

... is study was undertaken to simultaneously study the effect of vagal nerve stimulation ... he 5-HT levels in the portal circulation and on the cellular and subcellular morphology ... C in the cat.

Methods and materials

... Surgery, and blood sampling

... t cats of both sexes were used. Prior to the experiments the animals ... ere fasted for ... 4 h but had free ... s to water. Anesthesia ... as induced and maintained with pentobarbitone (30 mg/kg of b wt.). Blood ... ples (2.5 ml each) were drawn before, during, and after nerve stimulation according to the time schedule ... n in Fig. 1 through ... heparinized catheter inserted into the portal ... ein ... ith the tip in the hilus region. ... total volume of blood samples from each animal was 20 ml. For volume substitution all animals ... ere ... 20 ml of saline solution (0.9 ...) through the femoral vein before the initiation of electrical stimulation, ... an additional 20 ml ... ere given during stimulation. Blood samples were stored on ice during the ex... ments, centrifuged, and the plasma ... as kept deep frozen until the assay. Biopsy specimens of the ... denum ... ere taken from its first portion 1–2 cm apart immediately before ($n = 2$) and immediately after ... ion of nerve stimulation (... 2). Full-thickness specimens from 3 animals ... ere prepared for fluorescence ... roscopy; small mucosal specimens ... ere prepared for electron microscopy from all seven stimulated ... mals.

... e animal served as ... control and therefore had identical operations but no nerve stimulation. Blood ... ples ... ere drawn following the same time schedule.

... ve stimulation

... gi were dissected free at the cervical level and cut. The distal ends were placed in circular electrodes. ... outside of the vagi ... as insulated from surrounding tissues. A square ... ave impulse generator (Grass ... truments Co, Model S4) ... as used for the electrical stimulation. Both vagal nerves were stimulated ... multaneously for 15 min, ... ith supramaximal stimulation parameters within the following ranges, 8–20V ... 2ms, 6–10Hz. The effectiveness of nerv... stimulation was judged by increased bronchial secretion, small ... wel contractions, and urination.

... termination of 5-HT in plasma

... e portal blood samples (2.5 ml) were draw... initially and 1, 5, 10, and 15 min after onset of nerve stimula... on, then 5, 10, and 15 min after cessation of stimulation.

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The vagal release of 5-hydroxytryptamine from enterochromaffin cells in the cat

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Abstract

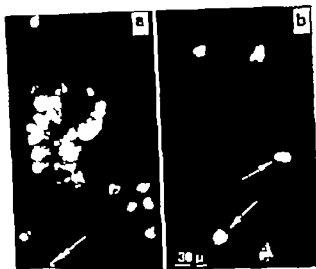
AHLMAN H., H N BHARGAVA, P E DONAHUE, B NEWSON T K DAS GUPTA and L M
NYHUS *The vagal release of 5-hydroxytryptamine from enterochromaffin cells in the cat*
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Enterochromaffin cells (EC) from the cat duodenum were studied before and after electrical efferent stimulation of the cervical vagi by means of electron microscopy and fluorescence histochemistry according to the Hillarp-Falck technique From the same animals portal blood plasma was assayed for serotonin (5-HT) at various stages of the nerve stimulation. Within 15 min after the onset of vagal nerve stimulation there was a more than twofold increase of the port l 5-HT levels, which returned to normal after cessation of stimulation. There was also a pronounced reduction in the fluorescence intensity of EC demonstrating lowered intracellula 5-HT level Ultrastructurally there was evidence for basal exocytosis as well as release of osmiophilic material from the EC directly into the gut lumen aft r nerve stimulation. Together these observations strongly suggest vagally induced 5-HT release from EC in the cat.

Key words Vagal nerve stimulation, enterochromaff n cells, 5-HT cat

Mammalian gut serotonin (5-HT) is located mainly in the enterochromaffin cells (EC) of the mucosa (Erspamer 1961 Penttilä 1966) In some species 5-HT is present also in mast cells (Enerbäck 1973). The presence of 5-HT containing neurons within the myenteric plexus has also been claimed (Gershon *et al* 1971 1976) EC are distributed all along the gastrointestinal tract, most frequently in the duodenum (Penttilä 1966, Forssman 1970). They are easily observed by fluorescence microscopy performed according to the Hillarp-Falck technique (1962) Their 5-HT content induces a bright yellow fluorescence at ultra violet illumination. By the use of cytofluorimetric techniques it now is possible to study the intracellular levels of 5-HT in individual EC (Ahlman *et al* 1976 a)

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Sweden.



2. Fluorescence micrograph longitudinal sections from ... glands (a) before and after vagal nerve stimulation. ... intense yellow fluorescence of ... has been markedly reduced ... in ... few cells (arrows). ... is the relative absence of ... fluorescent nerve terminals ... arrows) after stimulation.

...ctron-dense core of the granules. Only a few granules had a wide halo between the core ...d the limiting membrane in the resting state (Fig. 3).

The stimulated EC were partially degranulated. The majority of the secretory granules ...peared bereft of their dense osmophilia (Fig. 4 a), and many granules appeared with ...ide asymmetrical halos (Fig. 4 b). After the nerve stimulation the cytoplasm of the EC ...equently was enfolded, developing omegalike indentations close to the cell membrane. ...he secretory granules were frequently associated with these indentations, suggesting an ...tive process of basal exocytosis (Fig. 4 c). However it must be emphasized that the re...sponse was not all-or-none, and in some sections normal-appearing EC could be seen. Of ...terest was the unique coalescence of the secretory granules in the apical portion of the ...ells (Fig. 5). In the sections of stimulated mucosa, a progression of the secretory granules ...p to the most apical part of cells was observed. Also osmiophilic material could be dem...onstrated within the lumen of the gut (Fig. 6).

Discussion

The present study in the cat demonstrates a release of 5-HT into the portal stream during ...fferent stimulation of the cervical vagi. Such a release could be expected from earlier studies in which ... decrease of 5-HT was demonstrated in the duodenum, specifically in the EC, when studied by cytofluorimetry and light microscopy (Tansy ... *et al.* 1971 Ahlman 1976).

In the fluorescence microscope the major change after vagal nerve stimulation was the marked reduction in fluorescence intensity of the EC, due to a reduction of the intracellular ...evel, as described earlier by objective cytofluorimetric means in the same experimental ...en after atropinization (Ahlman *et al.* 1976 a, b). Occasionally however single ... seen with a moderately bright fluorescence, suggesting that all EC do not respond

The plasma concentration of 5-HT was determined by the modified fluorimetric method of Curzon Perlach (1973) for brain tissue Briefly the method consisted of vortexing 250 µl of plasma with n-butanol for 1 min followed by shaking for 5 min on mechanical shaker at 160 oscillations per minute The mixture was then centrifuged at 3 000 rpm for 5 min at 4 C. A 2.2 ml aliquot of butanol phase was transferred to tube containing 5 ml of n-heptane and 1.2 ml of 0.1 HCl containing 0.1 cysteine The mixture was shaken for 5 min at 160 oscillations per minute and then centrifuged at 3 000 rpm for 5 min 1 ml of aqueous phase was withdrawn and mixed with 0 4 ml of 0.012 o-phthaldialdehyde in 10 N HCl The mixture was heated in boiling water for 10 min. After cooling, the fluorescence of the sample was read at 470 nm after being excited at 360 nm on an Aminco-Bowman spectrophotofluorometer The recovery of 5-HT ranged from 72–81 per cent and the assays were reproducible within 5 per cent.

Fluorescence microscopy

The mucosal biopsy specimens were immediately frozen in liquid isopentane cooled by liquid nitrogen Thereafter the specimens were freeze-dried for 72 h and then reacted with formaldehyde gas at 80 C for 1 h according to the Hillarp-Falck procedure (cf Corrodi and Jonsson 1967). The formaldehyde gas was generated from paraformaldehyde equilibrated at relative air-humidities of 70 90 (Hamberger et al 1965). After paraffin embedding in vacuo the specimens were sectioned at 8–10 µm and mounted in xylene Entellane® (1 1) on heated glass slides. Fluorescence microscopy was performed with a Leitz microscope equipped for epillumination, using broad-band activation with filter combinations described earlier (Malmfors 1965). For photography Tri-X (Kodak) film was used with an exposure time of 60 s.

Electron microscopy

The 4 biopsy specimens from each cat were minced into 8 12 small mucosal specimens, which were immersed in 4 phosphate buffer glutaraldehyde at pH 7 4 for 4 h, while cooled on ice After this first fixation the specimens were postfixed with 2 phosphate buffer OsO at pH 7 7 for 2 h at room temperature dehydrated in acetones, then embedded in Araldite® Thick (1 µm) sections were stained with toluidine blue and used to orientate the sections. Thin sections (600 Å) were cut with a glass knife on a MT Porter Blum ultramicrotome then counterstained with uranyl acetate and lead citrate and viewed in RCA EMU 4 electron microscope Altogether about 250 different cells were studied. However a morphometric study was not performed.

Results

The efferent electrical nerve stimulation of the cervical vagi resulted in a marked increase of the 5-HT level in the portal vein plasma. Though the stimulation lasted for 15 min, the increase in portal 5-HT was most pronounced during the first 10 min. The increase in portal 5-HT was highly significant ($p < 0.001$) compared with the baseline level already after 5 min of nerve stimulation With cessation of nerve stimulation a rapid decrease in the 5-HT level was noted after 5 min, reaching baseline levels in 10 min. No changes of portal 5-HT were demonstrated in the nonstimulated, but shamoperated animal (Fig. 1)

When the duodenal specimens were studied by fluorescence microscopy before and after vagal nerve stimulation, distinct morphologic changes were seen The intensely bright yellow EC had, after stimulation, a pale yellow appearance. Furthermore, the 5-HT fluorophore also appeared to have diffused out through the cell membrane. Occasionally however single cells of moderately high fluorescence intensity were demonstrated in the stimulated specimens. The green fluorescent adrenergic nerve terminals surrounding the intestinal epithelium also appeared to be less intensely fluorescent after nerve stimulation (Fig. 2).

Ultrastructurally the mucosal EC were characterized by the presence of membrane-bound osmiophilic secretory granules in various shapes and varying densities, measuring from 100 nm to 600 nm. A symmetrical clear halo was often seen between the membrane and



Fig. 6. Section cut transversely to the luminal microvilli (mv) of two EC from stimulated animal. Arrows indicate osmiophilic granules in the gland lumen (lum). Note accumulation of characteristic granules in the apical region of both cells (EC_1 and EC_2). er = endoplasmic reticulum, m = mitochondria, d = desmosomes.

to the stimulus in the same manner. Since the prestimulatory and poststimulatory specimens from the same animal were reacted together in the same vessel during the histochemical procedure, differences in their fluorescence appearance should be true changes and not due to varying reaction conditions.

Ultrastructurally the most significant influence of vagal stimulation was the apparent degranulation of these secretory granules. In the resting phase the granules were filled with black or light gray material, but after stimulation the granules were more uniformly light gray or had wide halos, indicating partial release of the osmiophilic material. An emeiocytotic release of secretory material from gut endocrine cells has been earlier described after various types of stimulation (Fujita and Kobayashi 1971; Sano and Tobe 1977).

The fluorimetric assay of 5-HT demonstrated a release of this amine into the portal circulation during nerve stimulation. A direct release of the amine, first into the intercellular space and then to the capillaries, seems probable. However, this study also provides evidence of a vagal release of the osmiophilic material directly into the gut lumen as earlier demonstrated for somatostatin (cf. Uvnäs-Wallensten et al. 1977). If the osmiophilic material is identical with 5-HT, such luminal release of 5-HT has also been demonstrated in the perfused canine gut at catecholamine-(CA) infusion or splanchnic nerve stimulation by fluorimetric assay of the perfusate (Burks and Long 1966). Since the cervical vagus at the site of the stimulatory electrodes contains CA-nerve fibers running down to the gut (Ahl-











Fig. 3 Ultrastructure of an EC from nonst mulated nimal Characteristic granules of arious shape and electron densities, often w th a thin, symmetrical halo bet een the dense core and the limiting membrane (arrows). Empty vesicles and granules with wide halos are lso seen (double rrows).

Fig. 4 Ultrastructure of EC from the same n mal as Fig. 3 after vagal nerve st mulation. (a) EC close to the basal membrane with marked reduction of osmiophilia in the granules Si gle mpty esicles and wide-halo granules re also seen (double arrow). (b) Deta l of nother EC higher up in the same gland Many empty vesicles and wide halos are demonstrated (double-arrows). ( ) Detail of t EC close to the basal membrane of another gland. Granules appea t be relati ly unaffected in one of the cells (EC) However arrows indicate small clea vesicles and indentations f the outer membrane suggesting bed exocytosis in both cells (EC_1 and EC_2) Note also the ppare t increase n endoplasmic reticula (er), and the swollen mitochondria (m) cap = capillary in interstitial tissue

Fig. 5 Longitudinal section of n EC from a stimulated nimal. Not umerous ide halos among the basally located gra ules, but also the unique coalescence of osmiophilic mat pical ... cell (arrows).



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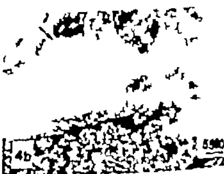






Fig. 3 Ultrastructure of an EC from nonstimulated animal. Ch racteristic granules of various shapes nd electron densities, often with a thin, symmetrical halo between the dense core nd the limiting membrane (arrows). Empty vesicles nd granules with wide halos are also see (double arrows).

Fig. 4. Ultrastructure of EC from the same animal as Fig 3 fter vagal nerve stimulation. ( ) EC close to the basal membrane with marked reduction of osmiophilia in the granules. Single empty vesicles and wide halo granules re also seen (double arrows). (b) Detail of another EC higher up in the same gland. Many empty vesicles and wide halos are demonstrated (double-arrows) ( ) Detail of two EC close to the basal membrane of another gland. Granules ppear t be relatively unaffected in one of the cells (EC_1). However arrows indicate small clear esicles and ind ntations of the out membrane suggesting brisk exocytosis in both cells (EC and EC_2). Note also the appare t ncrease in endoplasmic reticula (er), and the swollen mitochondria (m) cap = capillary in interstitial tissue

Fig. 5 Longitudinal section of an EC from a stimulated animal. Note numerous wide halos among the basally located gra ules, but also the unique coalescence of osmiophilic mat ... ical part of the cell (arrows).

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man *et al* 1978), the demonstrated vagal release of 5-HT might hypothetically be a [illegible]-mediated event The low fluorescence intensity of the adrenergic nerve terminals after stimulation also suggests that an adrenergic pathway to the gut might have been activated (Ahlman *et al* 1976 b) Whether 5-HT is released directly into the bloodstream or whether it is also released into the gut lumen is not clear If released directly into the lumen only, the prompt rise in portal 5-HT would reflect a rapid absorption of 5-HT from the lumen, perhaps aided by the local vasodilatory effect of the amine (Biber 1973).

The nerve stimulation was effective in all animals, judged by the changes in portal 5-HT levels however both fluorescence and electron microscopy revealed single cells with granules, which seemed to be unaffected. This might be due to local factors, but also suggests the possibility of separate populations of EC Fluorescence and electron microscopic studies have revealed at least two types of morphologically distinct populations of EC ([illegible] *et al* 1971 Gorgas and Böck 1976) Immunofluorescence studies have also demonstrated two populations of EC that store different polypeptides, such as substance P and motilin, in addition to 5-HT (Pearse *et al* 1974 1975). It is therefore possible that such populations may react differently to exogenous stimulation

A direct innervation of submucosal EC has been reported (Matsuo and Seki 1978). Recently a very close anatomic relationship has been described between nerve terminals, dendrites, and the mucosal EC, making a functional neural influence on those cells [illegible] (Lundberg *et al* 1977) The increase of 5-HT in the portal blood after vagal nerve stimulation might therefore reflect a direct neurogenic release. However intraluminal stimuli also have been reported to be effective in releasing 5-HT into the portal circulation Hyperosmolar sugar solutions, food, and duodenal acidification have proved to be effective in this respect (Smith 1958 Drapanas *et al* 1962, Wise *et al* 1968 Jaffe *et al* 1977). Such stimuli might act directly either on postulated luminal receptors of the EC or on sensory mucosal nerve endings, or cause release of intermediary mediator substances all possibilities leading to a final release of 5-HT from EC

5-HT has long been implicated in the physiology of the upper gastrointestinal tract. By using a very sensitive radioimmunoassay Kellum and Jaffe (1976) have demonstrated an increased peripheral blood level of 5-HT after a meal Biber (1973) earlier had suggested that 5-HT might be the transmitter in a postprandial vasodilation reflex. Other authors have emphasized the importance of 5-HT as a potent inhibitor in the feedback regulation of gastric acid secretion (Resnick *et al* 1962, Wise *et al* 1968, Jaffe *et al* 1977). It may seem somewhat contradictory that a procedure such as vagal nerve stimulation can activate gastric acid secretion and, conversely may lead to its inhibition by the release of 5-HT However it is possible that the gastric acid and 5-HT responses are mediated via two different types of efferent nerve fibers within the vagus (Ahlman 1976) which are activated simultaneously by the electrical stimulation in a nonphysiologic manner

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Influence of phenobarbital on changes in the metabolites of the energy reserve of the cerebral cortex following complete ischemia

By

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Abstract

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present study which concerns the rate of changes in the cerebral cortex concentrations of phosphocreatine (PCr), ATP, ADP, AMP, lactate and pyruvate during complete ischemia, had the objective of finding out whether or not phenobarbital retards depletion of tissue energy reserves during ischemia. Ischemia was induced for periods of 10 s to 10 min in animals maintained on 70% N_2O or given 150 mg of phenobarbital. The results showed that the barbiturate anesthesia delayed utilization of ATP during the first 2 min. However, after 5 min of ischemia PCr and ATP concentrations, as well as the calculated adenylate energy charge, were identical in animals anesthetized with nitrous oxide and phenobarbital. Thus, phenobarbital induces a very moderate delay in the depletion of cerebral energy reserves that occurs during complete ischemia. The results obtained after 5–20 s of ischemia allowed calculation of energy (~P) utilization according to Lowry *et al.* (1964). The closed system method gave values for ~P utilization which were not far from those obtained by CMR_{O_2} measurements. However, with normal values for metabolic rate (70% N_2O) valid estimates are obtained only with very short ischemic periods (5–10 s) and, with such short periods, the oxygen content of the tissue may introduce an error.

Although it is usually held that anaesthetics, and notably the barbiturates, retard changes in the metabolites of the energy reserve during complete cerebral ischemia, published results are equivocal. Lowry *et al.* (1964), in their comprehensive study of cerebral metabolic changes during ischemia, noted that phenobarbital slowed down anaerobic utilization of phosphocreatine (PCr) and ATP in 10-day old mice. The authors proposed a method for calculating preischemic energy (~P) flux from the rate of changes in glycogen, glucose, PCr, ATP and ADP (or from PCr, ATP, ADP and lactate) during the first 15 s of ischemia ("closed system method"). Phenobarbital, in a dose of 150 mg kg⁻¹ reduced ~P utilization to about 60% of the awake value. Later results from the same laboratory confirmed that phenobarbital delays energy depletion during the first 15–20 s of ischemia (Gatfield *et al.* 1966, Folbergrová *et al.* 1970; see also Brunner *et al.* 1971).

Subsequent studies on dogs have given results that are somewhat at variance with the reported in mice. Michenfelder and Theye (1970) induced ischemia by decapitation in animals under four anaesthetic circumstances, which provided a wide range of values for cerebral oxygen uptake (CMR_{O_2}) and measured rate of changes in tissue concentration of ATP and lactate. In all groups, there was a linear fall in ATP and a linear rise in lactate concentration during the first four min of ischemia. Irrespective of the type (or depth) of anaesthesia, there was no difference in rate of metabolic changes, but hypothermia reduced both the rate of fall in ATP and rate of rise in lactate concentration. On the basis of their results, the authors concluded that anaesthesia and hypothermia reduce metabolic rate by dissimilar mechanisms, and that anaesthetics cannot be expected to protect against the harmful effects of ischemia.

In the present study postischemic alterations in tissue concentrations of PCr ATP ADP AMP lactate and pyruvate were measured in paralysed rats that were either anaesthetized with 70 N O or with 150 mg kg of phenobarbital, employing ischemic periods varying between 5 sec and 10 min Since 70 N O does not significantly reduce CMR_{O_2} in paralysed rats (Carlsson *et al* 1976) the results allow evaluation of the "protective" effect of phenobarbital in complete ischemia.

Some of the present results have been reported in a preliminary communication (Nordström *et al* 1975) which was concerned with energy (~P) utilization, as calculated with the closed system method of Lowry *et al* (1964) Since this method has been extensively used for estimating metabolic rate in the brain a somewhat more detailed discussion of the results is given presently

Material and methods

The experiments were performed on fed male Wistar rats (300–400 g). The animals were divided into groups. In the first group anaesthesia was induced with 2 3 halothane to allow tracheotomy. Anaesthesia was then continued with artificial respiration using a gas mixture containing 70% N_2O and 30 O_2. The second group of animals was given 150 mg kg of phenobarbital *i.p.* After about 10-15 min, when the animals were deeply anaesthetized, they were tracheotomized and artificially ventilated on 70% N_2O and 30 % O_2. All animals were immobilized with tubocurarine chloride (1 mg kg^{-} *i.v.*) and were given heparin (100 I U *i.v.*).

In all animals a catheter was inserted into one femoral artery to allow continuous recording of arterial blood pressure and anaerobic sampling of blood Body temperature was kept close to 37°C and arterial P_{CO_2} and P_{O_2} were adjusted to 35–40 mmHg and 120–140 mmHg, respectively A left-sided craniotomy (10 15 mm) exposed the dura over the fronto-parietal cortex. Following operation all animals were left undisturbed for 20–30 min before the experiments were continued The exposed cortex was superfused with pre-warmed (37°C) artificial cerebrospinal fluid.

The cerebral cortex was frozen either without or with previous interruption of the cerebral circulation by decapitation, using isopentane pre-cooled to about 150°C in liquid nitrogen, which was allowed to superfuse the exposed dura The period of ischemia, the time between decapitation and freezing of the tissue, was either 5 10, 20, 60 or 120 s (N O anaesthesia) or 10, 20, 60 120, 300 or 600 (phenobarbital anaesthesia). Since speed of freezing is not critical extended periods of ischemia, values obtained after 5 and 10 min of ischemia in phenobarbital-anaesthetized animals were compared to previous data for animals maintained on 70 % N_2O (Ljunggren *et al* 1974). Control experiments showed that the superficial 1 mm of the cerebral cortex froze within 1–2 s. This part of the tissue was used for the chemical analyses.

The tissue samples were stored at −80°C until extraction. The analyses were performed with specific enzymatic fluorometric techniques (Lowry and Passonneau 1972) as described in detail in previous communications from this laboratory (Folbergrová *et al* 1972 a, b).

measured organic phosphates (PCr ATP ADP AMP) and substrates (pyruvate and lactate) were sed as μmol g^{-1} of wet tissue. The sum of the adenine nucleotides (ΣAd) was calculated as

$$\Sigma Ad = [ATP] + [ADP] + [AMP] \quad (\mu mol\ g^{-1}).$$

'energy state' of the tissue as calculated in terms of the adenylate energy charge (E.C.) according to son (1968):

$$EC = \frac{[ATP] + 0.5[ADP]}{[ATP] + [ADP] + [AMP]}$$

ergy (~P) utilization as estimated from changes in PCr ATP ADP and lactate according to Gat et al. (1966). Since glycogen concentrations did not change significantly in 20 (unpublished results), calculation should give alid estimate of ~P flux.
tistical differences ere evaluated ith Student's t-test.

Results

tissue concentrations of labile metabolites in animals anaesthetized with N_2O or phenobital are given in Table I and II, respectively In general, the results confirm many vious studies in showing that ischemia is accompanied by rapid reductions in the tissue centrations of PCr and ATP by a progressive rise in lactate concentration, by a transient rease in ADP and by a delayed and massive rise in AMP concentration (see .g. Lowry al. 1964, Gatfield et al. 1966, Gercken and Preuss 1969 Müller et al. 1970 Ljunggren al. 1974). An unexpected result was an increase in the sum of adenine nucleotides after 2 min of ischemia This occurred when the ADP concentration was maximally increased. ssibly the result is artefactual and is caused by the fact that the method for ADP is not ite specific (see Lowry et al. 1964)

One finding has not been recorded previously Thus, whereas Lowry et al (1964) observed rise in pyruvate concentration during the first 100 s of ischemia in 10-day old mice, anthetized with phenobarbital, the present results fail to demonstrate an increase in pyruate in adult rats, whether anesthetized with 70% N_2O or 150 mg kg^{-} of phenobarbital. n initial increase in pyruvate was recently observed also in 7-day old rats (Hansen and ordstrom, in preparation) which may indicate that the rise occurs only in juvenile aniials

The main results of the present study concern differences between the two anesthetic rcumstances, and calculated ~P utilization. These findings will be illustrated separately

Fig. 1 illustrates the rate of fall in PCr and ATP concentrations (in per cent of control), nd the reduction in adenylate energy charge. Phenobarbital anaesthesia somewhat delayed he fall in PCr concentration but, after 2 min, the PCr stores were virtually depleted in both roups. With 70 N_2O there was measurable decrease in ATP concentration already after 5 s, and the ATP was less than 10 of control after 2 min. In contrast, animals under henobarbital-anesthesia showed no fall in ATP concentration during the first 20 s, and about 50 remained after 2 min. However after 5 min ATP was close to zero in both anesthetic groups. The adenylate energy charge was reduced already after 10 s of ischemia in both nitrous oxide and phenobarbital groups During the first 2 min of ischemia the

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In the present study postischemic alterations in tissue concentrations of PCr, ATP, ADP, AMP, lactate and pyruvate were measured in paralysed rats that were either anaesthetized with 70% N_2O or with 150 mg kg^{-1} of phenobarbital, employing ischemic periods varying between 5 sec and 10 min. Since 70% N_2O does not significantly reduce CMR_{O_2} in paralysed rats (Carlsson *et al.* 1976) the results allow evaluation of the "protective" effect of phenobarbital in complete ischemia.

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Material and methods

The experiments were performed on fed male Wistar rats (300–400 g). The animals were divided into 2 groups. In the first group anaesthesia was induced with 2–3% halothane to allow tracheotomy. Anaesthesia was then continued with artificial respiration using a gas mixture containing 70% N_2O and 30% O_2. The second group of animals was given 150 mg kg^{-1} of phenobarbital *i.p.* After about 10–15 min, when the animals were deeply anaesthetized they were tracheotomized and artificially ventilated on 70% N_2 and 30% O_2. All animals were immobilized with tubocurarine chloride (1 mg kg^{-1} *i.v.*) and were given heparin (100 I.U. *i.v.*).

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[illegible] The lactate/pyruvate ratio during ischemia in [illegible]

Experimental group		PC	ATP	ADP	AM	Ad	LC	La	Py	La/Py
Control (n=4)		4.94 ±0.12	2.92 ±0.01	0.273 ±0.007	0.042 ±0.002	3.25 ±0.01	0.945 ±0.001	1.35 ±0.24	0.104 ±0.020	12.73 ±0.53
Ischemia (n=4)	10 sec	3.33 ±0.18	2.89 ±0.05	0.363*** ±0.039	0.054 ±0.001	3.31 ±0.06	0.929* ±0.003	1.64 ±0.07	0.080 ±0.009	20.63 ±0.70
Ischemia (n=3)	20 sec	2.27* ±0.07	2.92 ±0.09	0.373 ±0.007	0.054 ±0.002	3.34 ±0.09	0.928 ±0.003	2.78 ±0.07	0.063 ±0.005	44.10* ±2.40
Ischemia (n=4)	1 min	0.63 ±0.08	2.31 ±0.13	0.704*** ±0.112	0.218 ±0.075	3.44 ±0.03	0.835 ±0.036	7.22** ±0.33	0.095 ±0.010	79.3** ±10.0
Ischemia (n=4)	2 min	0.20* ±0.03	1.41 ±0.25	1.420* ±0.070	0.931 ±0.186	3.74 ±0.02	0.563 ±0.058	8.08 ±0.12	0.044 ±0.010	221 ±50
Ischemia (n=4)	5 min	0.06*** ±0.01	0.17*** ±0.02	1.122* ±0.026	1.976 ±0.013	3.27 ±0.06	0.224 ±0.004	10.83*** ±0.18	0.016 ±0.003	632*** ±90
Ischemia (n=4)	10 min	0.02* ±0.01	0.07* ±0.00	0.962 ±0.026	1.936 ±0.039	2.97** ±0.06	0.186 ±0.002	12.18 ±0.28	0.003 ±0.000	4849*** ±366

* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

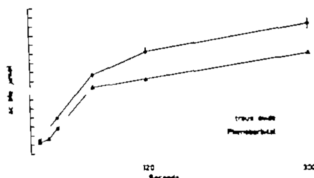
TABLE I Changes in PCr, ATP, ADP, AMP, lactate and pyruvate as well as in the calculated values for adenine nucleotide pool (ΣAd), energy charge (E.C.) and the lactate/pyruvate ratio during ischemia in animals anesthetized with 70% N_2O

Experimental group		PCr	ATP	ADP	AMP	ΣAd	E.C.	La	Py	La/Py
Control (n = 8)		4.74 ±0.09	2.91 ±0.03	0.294 ±0.013	0.050 ±0.006	3.25 ±0.04	0.940 ±0.003	1.50 ±0.14	0.125 ±0.009	11.89 ±0.41
Ischemia (n = 8)	5 sec	3.13 ±0.12	2.74 ±0.05	0.368 ±0.020	0.071 ±0.009	3.15 ±0.05	0.920* ±0.005	2.08 ±0.07	0.103 ±0.008	21.18 ±1.82
Ischemia (n = 8)	10 sec	2.35 ±0.13	2.72 ±0.05	0.405 ±0.025	0.076 ±0.012	3.21 ±0.03	0.913 ±0.008	2.76 ±0.16	0.089* ±0.005	31.04 ±1.02
Ischemia (n = 4)	20 sec	1.84 ±0.03	2.60* ±0.06	0.397* ±0.014	0.075 ±0.007	3.08 ±0.07	0.911 ±0.004	3.87 ±0.13	0.093 ±0.009	42.5 ±3.3
Ischemia (n = 4)	1 min	0.32 ±0.03	1.49* ±0.10	1.176 ±0.069	0.890* ±0.088	3.56 ±0.03	0.585 ±0.031	8.60* ±0.56	0.079 ±0.015	115 ±12
Ischemia (n = 4)	2 min	0.06 ** ±0.01	0.24 ±0.02	1.020* ±0.021	1.999* ±0.036	3.26 ±0.04	0.229 ±0.010	11.08 ±0.49	0.022 ±0.002	517* ±27
Ischemia	5 min	0.05 ±0.02	0.23 ±0.04	0.63 ±0.07	1.93 ±0.04	2.79 ±0.05	0.20 ±0.02	13.99 ±0.63	0.018 ±0.008	4 782 ±1 952
Ischemia	10 min	0.09 ±0.003	0.09 ±0.002	0.51 ±0.03	1.88 ±0.04	2.46 ±0.05	0.13 ±0.01	14.03 ±0.63	0.013 ±0.005	3 896 ±2 291

* $p < 0.05$ ** $p < 0.01$ $p < 0.001$

Values from LJUNGGREN *et al.* (1974). For these, there was a highly significant difference from control values ($p < 0.001$).

Increase in lactate content after 5, 10, 20, 60, 120 and 300 sec of ischemia in rats anesthetized with nitrous oxide or phenobarbital. The values are means ± S.E. Filled symbols denote values significantly different from controls (p < 0.05)



Discussion

...us freezing techniques have not been well suited to delineate metabolic changes ...ng after short periods of ischemia. This is due to the fact that when animals (or their ...) are immersed in a suitable coolant, there is an inevitable delay before the tissue is ... (see e.g. Swaab and Boer 1973, Ferrendelli *et al.* 1972, Pontén *et al.* 1973 a, b). Thus ...h instant the actual ischemic periods are longer than those given, and the error in... ...ced is directly related to the thickness of the insulating layers, and thereby to the size ... animals used. The present technique avoids this artifact since freezing was performed ...ugh the exposed dura (the circulation in the cortex being maintained) and since only the ...ficial 1 mm was used for analyses. It can be calculated that with this technique the ... analysed is frozen in about 1–2 s. The freezing technique does not alter the levels of ...y metabolites. The rapidity of freezing is, however, clearly demonstrated in the low ...ntage of phosphorylase *a* obtained (Folbergrová *et al.* 1978).

...fluence of phenobarbital in delaying depletion of energy reserves following ischemia

...present results amply confirm those of Lowry and associates (see Introduction) in ...ing that phenobarbital retards the rate of fall in energy reserves of the tissue following ...plete ischemia. Thus, whereas animals under 70% N_2O have less than 10% of their ... content left after 2 min of ischemia, phenobarbital-anesthetized animals still retain ...t 50% of the ATP pool at that time. However, if ischemia is prolonged to 5 min the ...ts are depleted of energy whether the animals are anesthetized with 70% N_2O or 150 ... kg^{-1} of phenobarbital. It may be asked if the alleged protective effect of the barbiturates ...schemia is at all related to the fact that they delay energy depletion within the first 5 ... In view of the fact that phenobarbital protects against the harmful effects of 30 min of ...ure ischemia although it cannot prevent energy depletion to occur within the first 5 min ...ordström *et al.* 1978), it seems necessary to invoke other explanations.

Evaluation of the closed system method for calculating metabolic rate

...ce the closed system method was introduced by Lowry *et al.* (1964) it has been used in a ... number of studies. The results have been quite variable and the method has never been ...idated against another technique for measuring metabolic rate. As discussed in a pre-

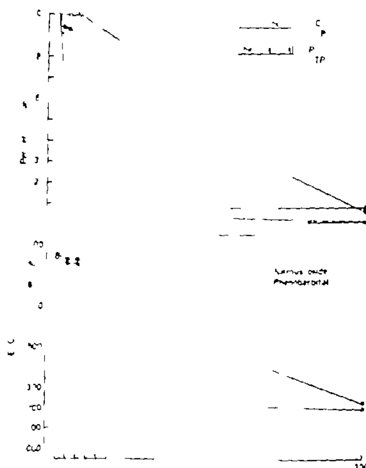


Fig. 1 Changes in energy ... the cerebral cortex after ... 60, 120 and 300 s of ischemia ... rats anesthetized with nitrous ... ide or phenobarbital. The ... figure shows the fall in PCr ... ATP (in per cent of control ... lower figure gives the change ... the adenylate energy charge ... Filled symbols denote values ... nificantly different from the con- trols ($p < 0.05$).

energy charge was significantly higher in the phenobarbital-anesthetized animals. How... no significant difference was seen after 5 min of ischemia.

Fig. 2 shows the rate of rise in lactate concentration. With 70% N_2O there was a ... rise in lactate at a rate of 7 μmol g⁻ min during the first min. Since normal gl... consumption in the cerebral cortex of the rat is 0.8 μmol g⁻ min (Borgström et al. ... the maximal rate of glycolysis during ischemia represents a 4- to 5-fold increase ... control. In the phenobarbital anesthetized animals, the rate of rise was delayed during ... first 10 s. However, in the following 50 s period lactate rose as it did in the nitrous ... animals. Thus, although phenobarbital retarded lactate production initially, it did not ... the peak rate of glycolysis. This finding confirms results reported by Lowry et al. (... The present results show that somewhat less lactate accumulated under phenobarb... anesthesia than under 70% N_2O (see Table I). In all probability, this is due to the fact ... in paralysed animals, phenobarbital anesthesia is usually accompanied by lower blood ... tissue) glucose concentrations than is anesthesia with 70% N_2O.

In summary, the present results demonstrate that phenobarbital anesthesia signific... retards utilization of high energy phosphate compounds during the first 2 min of isch... while this difference is no longer detectable if the period of ischemia is prolonged to ... to 10) min.

If the mean tissue oxygen tension is supposed to be 35 mmHg (see Mitnick and 1977) the amount of oxygen dissolved in brain tissue should be approximately 0.045 l^{-1} The total oxygen content of the blood and brain tissue should therefore be 0.045 0.11 μmol g⁻ The oxygen utilization rate of the rat cerebral cortex during l conditions is 4.5 μmol g⁻ min or 0.075 μmol g⁻ sec⁻ Thus, the calculated d of oxygen in the brain should suffice for 1.2 s of aer bic energy production. This only of importance when very short periods of ischemia are used for calculation of e of energy utilization, since the calculation indicates that energy derived from the stores could represent 20–40% of that used during 5 s of ischemia.

tentatively conclude that the closed system method of Lowry et al. (1964) gives ably accurate values for pre-ischemic energy flux when metabolic rate is reduced. At d metabolic rate, errors introduced by inexact timing of the period of ischemia, by ostischemic augmentation of metabolic rate, and by the oxygen content of the tissue, bute to render calculated values uncertain. It seems questionable that the method can plied to situations in which metabolic rate is increased.

thors are grateful to Maj-Lis Smith for skilful technical assistance. This study was supported by from the Swedish Medical Research Council (Project No. 14X 263) and from U.S. PHS Grant No. N507838 from N.I.H.

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TABLE III Rate of energy utilization in the rat cerebral cortex calculated from the closed system and from CMR_{O_2} measurements.

Anaesthesia	Number of animals	Time after decapitation (seconds)	~P utilization (μmol g⁻ min)	Energy flux ( ) calculated from CMR_{O_2} (μmol g^{-1} min^{-1})
N_2O	8	5	30±2	29
	8	10	4±2	
	4	20	17±1	
Phenobarbitone	4	10	11±2	15
	3	20	12±1	

liminary communication the present results allow such a validation in that CMR_{O_2} in the rat has been measured both in animals under 70 N_2O (Hägerdal *et al.* 1975) and in anesthetized with 150 mg kg of phenobarbital (Nilsson and Siesjö 1975).

Calculated rates of ~P utilization are given in Table III and are compared to those derived from measurements of CMR_{O_2}. In animals anesthetized with 70 N_2O the calculated after 5 or 10 s of ischemia fell close to those derived from CMR_{O_2}. However results indicate a gradual fall in ~P utilization with time of ischemia and, after 20 s value was significantly less than the 5 s figure. In contrast, phenobarbital anesthesia gave identical values for 10 and 20 s, somewhat lower than that expected from CMR_{O_2}.

The results of Table III corroborate the findings of Lowry *et al* (1964) that calculated ~P utilization falls off with time. In their study a linear rate of ~P use was obtained during the first 15 sec However our data indicate that "maximal values in N_2O anesthesia obtained only during the first 5 s. In contrast identical values are obtained after 10 and in animals anesthetized with phenobarbital Following the initial report of Lowry *et al* (1964) most subsequent studies have been carried out with ischemic periods of 15 s or longer (Gatfield *et al* 1966, Folbergrová *et al* 1970 Brunner *et al* 1971 Ferrendelli and C 1973). Probably the ~P values so obtained are too low because the ischemic interval been extended into the nonlinear part of the curve An exception is provided by the of Swaab and Boer (1972) who used the period 2–8 s and obtained a ~P utilization μmol g⁻ min) similar to the initial one reported here.

Even if the period of ischemia is kept short and well-defined by using a rapid freezing technique, the values of pre ischemic energy utilization rate obtained by the closed system method must be regarded with some caution. First, the method used for inducing ischemia may increase metabolic rate above normal (see Nilsson and Nordström 1977). Second metabolic rate is normal or increased very short ischemic periods must be used (see Third, since values close to those derived by a quantitative measurement of CMR_{O_2} obtained only when a very short (5 s) period of ischemia is used, the possible error introduced by the oxygen content of the blood and tissue must be considered. The oxygen content of cerebral blood in the rat can be assumed to be 6.7 μmol ml⁻¹ we may assume that each g of cortical tissue contains approximately 0.01 ml of (capillary) blood The solubility coefficient for O in brain tissue is 2.2 10⁻⁴) g atm (T

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From the Department of Neurology University of Goteborg, Sahlgren Hospital, Sweden

Effect of sympathetic stimulation on the blood brain barrier dysfunction induced by amphetamine and by epileptic seizures

By

BARBRO B. JOHANSSON and STAFFAN LUND

Received 1 March 1978

Abstract

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[illegible] I. Mean arterial pressure (MAP) before and after the increase of blood pressure (induced by amphetamine or bicuculline). $PaCO_2$ and pH before the sympathetic stimulation and pressure increase. Mean value ± SE. PaO_2 was > 13 3 kPa (100 mmHg) in all rats.

[illegible]imental groups		MAP before drug injection mmHg	MAP after	$PaCO_2$ kPa	pH
[illegible]etamine, control	5	138±3	186±6	4.8±0.1	7.37±0 01
[illegible]etamine, stimulation	5	139±4	189±9	4.7±0.2	7.37±0 01
[illegible]lline, control	7	144±3	190±3	4.7±0.1	7.37±0 01
[illegible]lline, stimulation	6	139±2	[illegible]02±5	4.7±0.1	7.36±0.01

[illegible] brains were taken for scintillation counting. Amphetamine induces leakage in cortical areas, predominantly in the anterior parts (Carlsson and Johansson 1978). The extravasation after bicuculline is more widespread but is usually most pronounced in the thalamus (Johansson and Nilsson 1977). Therefore, in the amphetamine groups the isotope content was determined in the cerebral cortex. In the bicuculline group protein leakage was also estimated in the thalamus and the cerebellum. Statistical differences [illegible] [illegible]ed with Wilcoxon test and Fisher' test.

Results

Rats given EB only 5 rats received amphetamine and 4 bicuculline during sympathetic [illegible]mulation MAP exceeded 175 mmHg in all rats and at this pressure level protein leakage [illegible] occurred in the brain in all normocapnic rats in earlier series with amphetamine or [illegible]culline.

In the amphetamine group 3 brains did not show any EBA leakage: a faint blue discolouration occurred in the other two brains (slightly more in the left, non-stimulated, hemisphere [illegible] one of these). In the bicuculline group some EBA extravasation took place at least in [illegible]e thalamus in all rats, but the leakage was less than is usually seen after a comparable [illegible]ood pressure increase. A slight side-to-side difference was seen in one rat only (left > right).

2. *Rats given both* [125]*IHSA and EB.* Table I presents MAP before and after the pressure [illegible]crease induced by amphetamine or bicuculline in the various groups. *Amphetamine* In [illegible]e control rats, EBA extravasation was moderate in 3 and marked in 2 brains. Two of the [illegible]rains from rats subjected to sympathetic stimulation did not show any EBA leakage: in

[illegible]able II. [125]IHSA leakage in the brain after an amphetamine-induced blood pressure increase in rats subjected to stimulation of the right cervical sympathetic chain and in non-stimulated controls. The tracer content is expressed as 100·(count $min^{-1}\cdot mg^{-1}$ brain tissue)/(count $min^{-1}\cdot mg^{-1}$ blood). Mean ± SE.

Experimental groups	Right cerebral cortex	Left cerebral cortex
Control (n 5)	0.38±0.11	0 37±0 08
Stimulated (n 5)	0 12±0 03*	0.12±0 05

[illegible] P 0 05 for significant differences from the controls.

administration of amphetamine these models can be used to study the effect of increased intraluminal pressure on the BBB As regards the cerebral metabolism there are important differences. Thus, the cerebral oxygen consumption is not increased in acute hypertension *per se* whereas the metabolic rate is greatly enhanced in bicuculline-induced seizures (Meldrum and Nilsson 1976) and is increased also after the administration of amphetamine (Carlsson Hägerdal and Siesjö 1975 McCulloch and Harper 1977)

The BBB studies mentioned so far have been performed on anesthetized animals. Acute angiotensin-induced hypertension can induce BBB dysfunction also in conscious rats (Johansson and Henning 1976) However in a recent comparative study it was found that awake rats developed less BBB dysfunction than anesthetized rats when they were exposed to an abrupt rise in blood pressure (Johansson 1978) This was particularly evident when the pressure increase was induced by amphetamine or by bicuculline.

Bill and Linder (1976) have demonstrated that stimulation of the cervical sympathetic chain during acute hypertension prevents the blood flow increase and break-down of the BBB on the stimulated side. Studies by Edvinsson, Owman and Siesjö (1976) and MacKenzie *et al* (1977) have confirmed that a shift in the upper limit of the autoregulation toward higher absolute levels of arterial pressure occurs during sympathetic stimulation. The experiments suggest that one possible explanation to the difference between conscious and anesthetized animals to pressure-induced BBB dysfunction could be a difference in sympathetic tone The present study was performed to see if unilateral stimulation of the sympathetic cervical chain could attenuate or prevent the BBB dysfunction when the blood pressure increase was combined with a metabolic vasodilatation

Material and methods

Male Sprague Dawley rats (350–400 g) were used. Anesthesia was induced with diethylether the rats tracheotomized immobilized with suxamethonium chloride and artificially ventilated with a mixture 30% O and 70% N_2O A femoral artery was cannulated for anaerobic sampling of blood and for recording of the mean arterial pressure (MAP), a femoral vein was cannulated for intra venous (i.v.) injection of drugs and tracers. PaO_2, $PaCO_2$ and pH were determined and only normocapnic rats with PaO_2 > ... mmHg were included in the study 2 Evans blue (EB) in saline (1 ml kg^{-1}), which binds to albumin, was given i.v. before the injection of bicuculline or amphetamine to enable visual inspection of protein extravasation. The body temperature was maintained at approximately 37°C.

The right cervical sympathetic chain was isolated and sectioned approximately 1 cm below the upper sympathetic ganglion. The cranial end of the nerve was mounted on bipolar silver electrodes with the proximal electrode being cathod. The nerve and the electrodes were further surrounded by liquid paraffin to prevent drying of the nerve. Square wave pulses of 1 ms duration at 10 Hz were used. The threshold voltage was determined by observing the dilatation of the pupil and the appearance of exophthalmus on the homolateral side and 10 times this voltage was used for stimulation (maximum voltage used 1 V). The vagus nerve was usually left intact (in two experiments the nerve was sectioned for technical reasons). d l-amphetamine sulphate (2 5 mg kg^{-1}) or bicuculline (1 2 mg kg^{-1}) was injected i.v. 30 s after the beginning of the stimulation, which continued until the animal was killed by i.v. pentobarbital 3 minutes later The extravasation of Evans blue-albumin (EBA) was registered and compared in the two hemispheres.

Because of the preliminary results from these series, an apparent bilateral effect of unilateral stimulation (see results), the experimental design was changed as follows ^{125}I-serum albumin (^{125}IHSA) was given in addition to EB to allow a detailed comparison of the extravasation in the two hemispheres and between stimulated and non-stimulated animals. Amphetamine or bicuculline was injected either during sympathetic stimulation or to non-stimulated controls. Three minutes later the brains were briefly perfused in situ with saline (30 seconds) to remove tracer-containing blood from the cerebral vessels. Predetermined areas of

l of blood pressure. Bilateral changes of cortical blood flow and of the diameter of pial vessels were also observed in some normotensive cats by Kobayashi, Waltz and Rhoton (1971). The extensive literature on the effect—or lack of effect—on the CBF by sympathetic stimulation in normotensive animals has recently been reviewed by Edvinsson and MacKenzie (1976) and will not be further discussed in the present paper

The sympathetic innervation of cerebral vessels is basically unilateral (see Edvinsson 1975). In the rat, the two anterior cerebral arteries unite to form an unpaired anterior cerebral trunk (Zeman and Innes 1963). Thus, constriction of one of the supplying arteries could reduce the blood flow also to the contralateral side and might help to explain the effect of sympathetic stimulation in the amphetamine model, in which the most severe extravasation usually takes place in the anterior part of the brain (Carlsson and Johansson 1978). However, this fact alone does not satisfactory explain the functional bilateral protection of unilateral stimulation found in the present series and by MacKenzie *et al.* (1977)

The difference between our results and those of Bill and Linder (1976) might possibly reflect species difference. Some differences in the techniques applied may also have played a role. Bill and Linder cut the vagus nerve on the stimulated side and on the contralateral side both the sympathetic trunk and the vagus were sectioned, a procedure which might have accentuated a side difference. In the present study these nerves were left intact.

The vasodilatation seen in acute hypertension is mechanically induced whereas the vasodilatation induced by amphetamine is thought to be—at least in part—secondary to a catecholamine-induced increase of cerebral metabolism (Carlsson, Hägerdal and Siesjö 1975 McCulloch and Harper 1977 Carlsson and Johansson 1978). There is a functional coupling between the increased metabolism and blood flow in experimental seizures but the trigger mechanism behind the vasodilatation is currently under debate and is probably complex (see Astrup *et al.* 1976). It might seem surprising that sympathetic stimulation can influence metabolically induced vasodilatation. The effect of sympathetic stimulation is not necessarily an exclusive effect on the intracerebral vessels. It has been suggested that the effect of sympathetic stimulation is primarily an effect on the carotid artery and its larger extraparenchymal branches Constriction of these arteries would normally lead to dilatation of the vessels distal to the constriction because of autoregulation of the cerebral blood flow. However in conditions with maximally dilated intracerebral vessels it would decrease CBF. This hypothesis has been proposed to explain the reduction in flow observed in hypercapnic animals during sympathetic stimulation (James, Millar and Purves 1969 Kobayashi, Waltz and Rhoton 1971 Harper *et al.* 1977). The greatest reduction in flow was seen at a high PCO_2 when the initial blood flow was the highest.

In conclusion, our results indicate that the prophylactic effect of sympathetic stimulation on the BBB in acute hypertension—first demonstrated by Bill and Linder (1976)—is at hand also when hypertension is combined with metabolically induced vasodilatation. It is not clear whether the sympathetic nerves exert an influence predominantly on the intracerebral or extracerebral vessels or if it is a combined effect on both.

Excellent technical assistance was given by Barbro Eriksson and Ulla Kruse. The study was supported by grants from the Swedish Medical Research Council (project No. 14X-4968) and from the Swedish National Association against Heart and Chest Diseases

TABLE III Extravasation of ^{131}IHSA in the brain after the administration of bicuculline [illegible] concomitant stimulation of the right cervical sympathetic chain and to non-stimulated [illegible] The tracer content is expressed as 100 (counts min mg^{-1} brain tissue)/(counts [illegible] blood). Mean ± SE.

Experimental groups	Cerebral cortex		Thalamus		Cerebellum
	right	left	right	left	
Control (n = 7)	0.59 ± 0.17	0.56 ± 0.16	0.81 ± 0.29	0.85 ± 0.32	[illegible]
Stimulated (n = 6)	0.16 ± 0.02	0.19 ± 0.03	0.51 ± 0.05	0.58 ± 0.05	[illegible]

$p < 0.01$ for significant differences from the controls.

two brains a faint and in one brain a moderate blue staining was seen. ^{131}IHSA content in cortical areas from the stimulated and non-stimulated side is presented in Table II. In agreement with the visual estimation of EBA extravasation, no difference was observed between the right and left hemisphere (In comparison it should be mentioned that the corresponding tracer content in normal rat brain, *i.e.* from rats not subjected to hypertension: 0.02–0.04 see Carlsson and Johansson 1978). *Bicuculline* In the controls the pattern and degree of EBA extravasation was in agreement with earlier series, *i.e.* protein leakage occurred in many regions including the cerebral cortex but was most pronounced in the thalamus. Some blue staining was present in the thalamus also in the stimulated rats (left side slightly more than right in two brains) the EBA leakage in the cortical areas was slight or absent. The fact that bicuculline increased MAP more in the stimulated group than in the controls (see Table I) would *per se* be expected to result in a higher degree of protein extravasation and explains why the protein leakage from the cerebellar vessels, which have a sparse sympathetic innervation (Edvinsson 1975) was more pronounced in the stimulated group than in the controls (see Table III). The effect of sympathetic stimulation was stronger on cortical resistance vessels than on vessels in the thalamus. The difference in protein leakage in the thalamus between stimulated rats and controls was not significant.

Discussion

The bilateral preventive effect on the BBB function produced by a unilateral sympathetic stimulation was unexpected. A slight difference between the stimulated and non-stimulated side was noticed in a few rats. The only marked side-to-side difference we have observed was in a rat given amphetamine which was excluded from the series presented here because it was hypercapnic (*cf* Johansson and Nilsson 1977) Bill and Linder (1976) reported a marked side difference as regards both CBF and BBB function between the stimulated and non-stimulated side in their experiments on acute hypertension in cats. On the other hand MacKenzie *et al* (1977) have found a bilateral effect on the CBF by unilateral stimulation in acutely hypertensive baboons. They found no consistent side to side difference at any

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strogen induced inhibition of ³H-noradrenaline release in the uterus and portal vein of the rat

By

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Abstract

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e influence of estrogen treatment on ³H-noradrenaline release, induced by potassium or calcium, as died in isolated preparations of the uterus and the portal vein of the rat. Uterine strips of oophorectomized rats responded with contraction followed by transient relaxation when immersed in medium containing 127 mM potassium. The transient relaxation was accompanied by an increased rate of ³H-noradrenaline efflux. When the uterine strips were bathed in calcium-free potassium solution, addition of calcium (3 mM) evoked an increased outflow of ³H-noradrenaline coinciding with relaxation phase of contractile response to calcium. After estrogen treatment of the rats the pattern of response was altered. uterus then responded to potassium and calcium with sustained contractures and with relatively all (potassium) or with no (calcium) increase of ³H-noradrenaline efflux. Normetanephrine (5 · 10⁻⁶ M) not influence the uptake or release of ³H-noradrenaline in either non-estrogenized or estrogen treated uterus. Calculations, based on the amounts of ³H-noradrenaline released by potassium and on the inhibitory effects of desipramine (10⁻⁶ M) on neuronal uptake of noradrenaline, suggested that similar amounts of tracer were accumulated in the adrenergic nerves of both non-estrogenized and estrogen treated preparations. Tyramine (10⁻⁴ M) had weaker stimulatory effect on ³H-noradrenaline release than potassium, and the tyramine induced release was not inhibited by estrogen treatment. Strips of the rat portal responded to potassium with an increased rate of ³H-noradrenaline efflux. As in the uterus, estrogen treatment reduced the release of ³H-noradrenaline in response to potassium. The adrenolytic effect of estrogen is possibly due to reduced entry of calcium ions into the nerve terminal.

vidence has previously been presented that smooth muscle contractures, induced by potassium or calcium, are under certain conditions modified by the release of intramural noradrenaline (Bengtsson 1977 a, 1978). Thus the contractures of the rat uterus exhibited a transient relaxation that did not appear in a reserpinized preparation or after β-adrenoceptor blockade. The contractile response of the rat portal vein, on the other hand, was enhanced by the release of endogenous noradrenaline. An additional finding was that estrogen treatment completely (uterus) or partially (portal vein) inhibited the contractile effects of intramural noradrenaline. This sympatholytic effect of estrogen could not be accounted for at least not on a quantitative basis, by decreased sensitivity of the adrenoceptors, suggesting that estrogen might reduce the amount of noradrenaline released.

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...B However estrogenized strips were not divided longitudinally and no ¹⁴C-sucrose was added to ...ubation medium. Each specimen was transferred through a series of 4 tubes containing tracer-free ... The first 3 tubes of each series contained Na-Krebs. The last tube contained K Krebs in 3 of the ... and tyramine in Na-Krebs in the 4th one. The time schedule for bathing the tissues was 10 10 ...ds. Drugs used, except tyramine, were present in the solutions from the beginning of the equilibra... ...riod to the end of the expt.
...of vas. The experimental design was essentially the same as that described for the uterus under ...tion B.

...ination of ³H-noradrenaline and ¹⁴C-sucrose The preparations were weighed before mounting on ...ed tubes and dissolved in solubilizer as described by Batra and Bengtsson (1976), at the end of ...pt. A toluene-[Triton-X-100]-mixture was used as scintillation-fluid (Patterson and Greene 1965). ...adioactivity was measured in liquid scintillation counters (Packard TriCarb or Nuclear Chicago ...300. Quenching was corrected for by using the external standard method.
...calcium. The contractile responses were read directly from the original recordings whereas the ...of the isotope measurements have been treated as follows. In all expts. the ³H-noradrenaline content ...f the tissue at any given time was calculated by adding the total amount of ³H-noradrenaline released ...this time to that remaining in the tissue at the end of the expt. In the A and B-series and in the expts. ...portal vein, efflux of ³H-noradrenaline is expressed as rate coefficient (r) in min⁻¹

$$\frac{A}{\Delta t \cdot Q}$$

...A represents the radioactivity lost in the time interval Δt, and Q is the content of ³H-noradrenaline ...tissue at the midpoint of the time interval Δt. In the C-series the rate of ³H-noradrenaline efflux is ...ssed as mol min⁻¹ (A/Δt). Further details on calculations are given under Results ...statistical significance was determined by the use of Student t-test.

...ions

...muscles were bathed in Krebs solution (Na-Krebs) of the following composition (mM): NaCl 119 ...4.6, $NaHCO_3$ 20, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 11 pH 7.4. When the effects of de... ...ization were studied an isotonic potassium solution (K-Krebs) of the following composition was ...(mM): KCl 127 $NaHCO_3$ 20, $CaCl_2$ 1.5, NaH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 11 pH 7.4. In the calcium-... potassium solution, $CaCl_2$ was omitted and EGTA (0.01 mM) was added. When effects of calcium ...studied concentrated solution of $CaCl_2$ was added to the calcium-free potassium solution (final ...3 mM). EDTA (3 10⁻⁵ M) and sodium ascorbate (5 10⁻⁴ M) were added to the media to prevent ...ation of noradrenaline. The solutions were equilibrated with a mixture of 5 CO_2 and 95 O_2 and ...at 37 C

...gs and isotopes

...(Ciba), normetanephrine (Sigma) and tyramine (BDH). DL-noradrenaline-7-³H (9.9 Ci ...ol) and sucrose-¹⁴C (5 mCi/mmol) (New England Nuclear Corporation).

Results

Uterus

Contractile responses and release of ³H-noradrenaline

...In Na-Krebs solution the uterus contracted spontaneously. When this medium was re...placed by K Krebs containing 1.5 mM calcium, a brief myometrial contraction was followed ...by a pronounced relaxation (Fig. 1). The muscular tension transiently increased when the ...calcium containing K Krebs was replaced by calcium-free potassium solution. Addition of ...mM calcium to the isotonic potassium solution caused a phasic increase in tension fol-

It has been the aim of the present investigation to further characterize the release of intramural noradrenaline during potassium and calcium induced contractures of smooth muscle. To this end the time course of potassium and calcium induced release of noradrenaline has been studied and correlated with the mechanical events in the uterus and portal vein of the rat. It has also been investigated whether the sympatholytic effect of estrogen is mediated through inhibition of noradrenaline release from the adrenergic nerves or if increased extraneuronal binding prevents the noradrenaline from acting on the alpha-receptors. A preliminary report on this investigation has been given previously (Bengtsson 1977 b)

Methods

Animals

46 virgin female rats of the Wistar strain, weighing 150–200 g were used. All animals were oophorectomized and half the number were given 0.5 mg of polyestradiol phosphate i.m. immediately after operation. The dose (related to body weight) of polyestradiol phosphate is about double that used clinically to treat menopausal symptoms. There were close similarities between estrogen treated and estrus rats and non-estrogenized and diestrus rats, respectively as to macroscopical appearance of the uterus. Corresponding similarities were found when calcium contractures were evoked in the depolarized uterus (Bengtsson 1978). Estrogen treated rats were killed on day 4 and the rest of the animals (non-estrogenized) were used on day 11 after operation. Myometrial contractures evoked by potassium or calcium displayed the same pattern on day 4 as on day 7

Preparations and experimental procedures

Either the uterus or the portal vein were isolated and placed in Na Krebs solution after which they were split open (the uterus at the mesenteric border) longitudinally

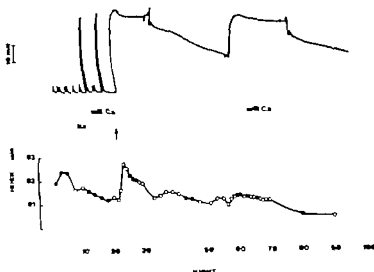
Uterus. Procedure A. About 20 mm of the middle part of one of the uterine horns was used. This was mounted longitudinally in a 2 ml jacketed organ bath containing Na Krebs and subjected to a tension of 10 mN which within 10 min subsided to a steady level at 1.2 mN. After an accomodation period of 30 min the tissue was incubated with ^{3}H-noradrenaline (4 $\cdot$ 10^{-7} M) and ^{14}C-sucrose (3 $\cdot$ 10^{-3} M) in Na Krebs

^{14}C-sucrose was used as an extracellular marker in order to investigate whether tracer substance could be squeezed out of the tissue as a result of the contraction. When the tissue was contracted by potassium a small increase of the rate constant of ^{14}C-sucrose efflux could be seen in some expts. However this increase was less than 1/10 of that seen for ^{3}H-noradrenaline on stimulation with potassium.

After 60 min of tracer uptake the uterus and the bath were rinsed three times with non-radioactive Na Krebs (total time for rinsing 15 s). Then the muscle chamber was continuously perfused at constant rates (0.5–2 ml/min) with isotope-free solution which were collected in 2 ml samples through a outlet at the top of the bath. Mixing was obtained by bubbling the fluid with 95% O_2 and 5% CO_2 from the bottom of the bath. The contractile activity of the muscles was recorded isometrically by a tension transducer (Grass FTO3-C) connected to an ink-writer (Beckman R 611).

Procedure B. About 20 mm of the middle part of one uterine horn was used. Estrogenized strips were divided longitudinally in order to reduce the weight difference between untreated and estrogenized preparations. After this modification the weight of the estrogenized preparations varied between 46 and 65 mg whereas the weight of untreated tissue was 43–48 mg. The strips were mounted on tubes of stainless steel, stretched to their natural length, and placed in Na Krebs for an equilibration period of 30 min. The fluid was stirred by bubbling with 95% O_2 and 5% CO_2 through the steel tubes. After equilibration the suspended strips were placed in plastic tubes containing 3 ml Na Krebs with 4 $\cdot$ 10^{-7} M ^{3}H-noradrenaline and 3 $\cdot$ 10^{-3} M ^{14}C-sucrose. The incubation with isotopes lasted for 60 min after which tissues and steel tubes were rinsed 3 times in 100 ml non-radioactive Na Krebs (total rinsing-time 15 s). The preparations were then transferred through a series of plastic tubes each containing 3 ml of tracer-free solution. The tissue was bathed for 10 ml in each tube. Isotopes recovered in the bathing solution of these tubes were measured by counting radioactivity in 2 ml aliquots.

Procedure C. Both uterine horns were used. They were divided by transverse section into 2 pieces of about equal length, giving 4 strips from each uterus. These strips were essentially treated as described



Mechanical activity (upper trace) and rate coefficient of ³H-noradrenaline efflux (lower trace) in ...rized rat uterus. Perfusing media as indicated between the traces. Na^+ = Na-Krebs, K = K Krebs, ...indicates shift from Na to K.

...ased the contracture tension in estrogen treated uterus. No significant spontaneous ...ation was observed in this case, in line with the finding that calcium did not cause any ...ase of the ³H-noradrenaline release.

...uantitative analysis of the release of ³H-noradrenaline induced by ...tassium and calcium

...results shown in Fig. 1 and 2 indicated that both potassium- and calcium-induced ...adrenaline release in the rat uterus was impaired by estrogen treatment. In order to ...ntitate the ³H-noradrenaline release, the latter was studied in tissues (7 untreated and ...trogenized rats) which were exposed to Na-Krebs, K Krebs and increased calcium ...centration in the same order as in the expts. described under A. The mean rate coefficient ...tracer efflux into these solutions is shown in Fig. 3. The rate constant of ³H-noradrenaline ...ux in K-Krebs containing 1.5 mM calcium (third column) was 0.038 ± 0.001/min in the ...treated and 0.019 ± 0.002/min in the estrogenized tissue. When the noradrenaline release ...s stimulated by 3 mM calcium (last fraction) the mean rate coefficient of efflux was ...29 ± 0.001/min in non-estrogenized uterus whereas the corresponding value for estro...nized preparations was only 0.014 ± 0.001/min. In consequence there remained a larger ...ction of ³H-noradrenaline in the estrogenized (0.246 ± 0.018) than in the untreated tissue ...198 ± 0.007) after the washout period.

... Effects of desipramine, normetanephrine and tyramine on uptake and ...release of ³H-noradrenaline

...he data presented in the previous sections suggest that the release of ³H-noradrenaline ...om adrenergic nerve endings was reduced by estrogen treatment. However the reduction

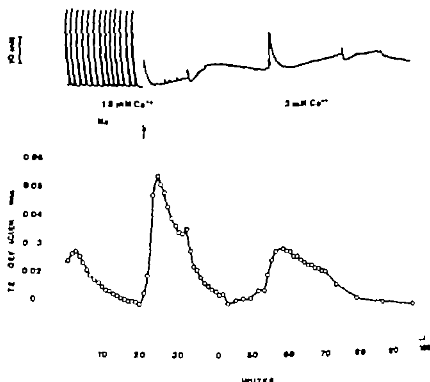


Fig. 1 Mechanical activity (upper trace) and rate coefficient of ³H-noradrenaline efflux (lower ... non-estrogenized rat uterus. Perfusing media is indicated between the traces. Na = Na-Krebs, K = K-Krebs, arrow indicates shift from Na to K

lowed by a slowly developing contracture When calcium was again omitted from the ... the preparation relaxed slowly

In the lower part of Fig. 1 the simultaneous efflux of H-noradrenaline has been plotted as rate coefficient against time. The rate coefficient declined from an initial value of approximately 0 025/min to 0 009/min within the first 20 min of the washout period ... had no visible effect on the contraction When the bathing medium was changed from Na Krebs to K Krebs the rate coefficient of H noradrenaline efflux increased and reached a maximum of 0 054/min which coincided in time with the spontaneous relaxation phase of the potassium contracture. The efflux rate then declined and this was paralleled by partial restoration of the contractile tension After omission of calcium from the extracellular medium the rate constant of noradrenaline efflux continued to decrease and a final value of about 0 01/min was reached. Addition of calcium (3 mM) to the potassium solution evoked an increased outflow of H-noradrenaline coinciding with the relaxation phase of the calcium induced contracture

Estrogen treatment of the rats modified the contractile responses of the isolated ... (Fig. 2, upper tracing) The spontaneous activity in Na Krebs was generally infrequent ... irregular in the estrogenized myometrium. In this preparation K Krebs produced a sustained contracture and it can be seen that there was a relatively small release of H-noradrenaline in this case. Omission of Ca from the bathing fluid caused a decline of the contractile tension, but had very little effect on the ³H-noradrenaline release. Addition of calci...

Effect of drugs on uptake and release of ^{3}H-noradrenaline in rat uterus

Treatment	Weight of uterine strips (mg)	^{3}H-noradrenaline in uterine strips before release (pmol)	Releasing agent	^{3}H-noradrenaline released by K or tyramine (pmol/min)
Non-estrogenized (n 6)				
Control	28.5 ± 1.4	52.3 ± 3.1	K	4 00 ± 0 38
Desipramine 10^{-6} M	28.3 ± 2.2	9 1 ± 1 3	K	0 21 ± 0 14
Normetanephrine 5 10^{-4} M	27.8 ± 2 1	51.3 ± 3.7	K	3 76 ± 0 20
Normetanephrine 5 10^{-4} M	26 2 ± 2 6	37 7 ± 3.1	Tyramine 10^{-4} M	0.31 ± 0.07
Estrogen treated (n = 6)				
Control	86 5 ± 10.4	59 9 ± 2 5	K	1 49 ± 0 28[b]
Desipramine 10^{-6} M	82.2 ± 7.3	31 1 ± 2 9	K	0.42 ± 0 12
Normetanephrine 5 10^{-4} M	80 8 ± 7 7	58.3 ± 4 0	K	2 06 ± 0.33[b]
Normetanephrine 5 10^{-4} M	81.0 ± 7 9	51.8 ± 3 9	Tyramine 10^{-4} M	0 47 ± 0.10

uterine strips were from the cervical end in the control and normetanephrine-K series and from the ...n end in the desipramine and normetanephrine–tyramine series
...ms from non-estrogenized p 0 01.

...was a considerable weight difference between the groups, the estrogenized prepara... weighing 3-4 times as much as the untreated ones.

...tissues were exposed to potassium or tyramine after 21 min of efflux in Na-Krebs. ...is time most of the ^{3}H-noradrenaline in the extracellular fluid had left the tissue ac...ing to expts. in which ^{14}C-sucrose was used as a marker for extracellular binding (see ...ods). The calculated amounts of ^{3}H-noradrenaline existing in the preparations im...ately before they were subjected t potassium or tyramine are given in Table I In ...ese expts. the main question was whether a given number of adrenergic nerves in the ...s would respond with reduced transmitter release after estrogen treatment. For this ...on the tissue contents and the amounts of released ^{3}H-noradrenaline have not been ...malized with respect to tissue weight as this would produce seemingly small values in ...estrogenized uterus (cf above). The estrogen treated tissues contained somewhat larger ...unts of tracer than the non-estrogenized preparations after immersion in the control ...ution. After inhibition of neuronal uptake by desipramine the amount of ^{3}H-noradrena... was reduced considerably in the non-estrogenized and t lesser degree in the estro...ized uterine strips. Normetanephrine, on the other hand, did not influence the contents ...of ^{3}H-noradrenaline in either non-estrogenized or estrogen treated tissue. The difference ...ween the tracer contents in the two series of normetanephrine treated preparations (cf ...rows 3 and 4 and 7 and 8, respectively) is probably explained by the fact that the cervical ...end of the rat uterus, used in the normetanephrine-K series, has more dense adrenergic ...innervation than the ovarian end (Adham and Schenk 1969 Hervonen et al. 1973), used ...in the normetanephrine-tyramine series.

Table I also shows the effects of potassium and tyramine on the rate of ^{3}H-noradrenaline

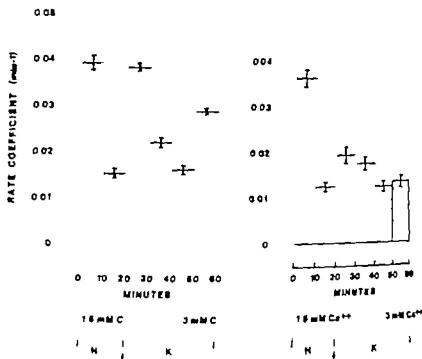
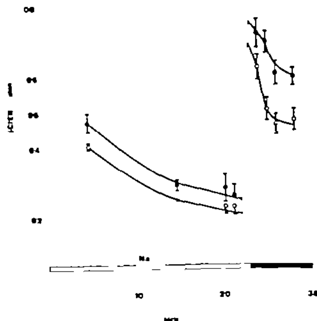


Fig. 3 Histograms showing rate constants of H-noradrenaline efflux from non-estrogenized (left) and estrogen treated (right) rat uterus. The preparations were consecutively bathed for 10 min in media of composition indicated below the graphs. Na = Na-Krebs, K = K Krebs, arrow indicates shift from Na to K. Values are mean ± S.E. of 7 (non-estrogenized) and 6 (estrogen treated) expts.

of the efflux coefficient in the estrogenized uterus might also be due to other mechanisms. There is a pronounced hypertrophy of uterine tissue without a concomitant increase in the amount of noradrenaline in the rat uterus under estrogen treatment (Oskarsson & Falck *et al* 1974). Thus the possibility existed that the extraneuronal fraction of noradrenaline was much larger in the estrogen treated uterus, and that a spuriously low value of the potassium or calcium-induced neuronal release therefore was obtained in this tissue. In order to examine this possibility the uptake and the release of H-noradrenaline was investigated in the presence of 1 desipramine which selectively blocks neuronal uptake (Titus and Spiegel 1961 Sigg *et al* 1963) and normetanephrine which has been shown to inhibit extraneuronal uptake (Burgen and Iversen 1965 Eisenfeld *et al*. 1967). Another means of clarifying the estrogenic effect on H-noradrenaline release was to induce release of noradrenaline with an agent different from potassium and calcium. For this purpose tyramine was chosen, as this substance has been shown to release noradrenaline by a process that is independent of calcium (Lindmar *et al* 1967). The tissue uptake and release were followed in drug free solution (control) and in solutions containing desipramine (10^{-6} M) or normetanephrine (5 10^{-6} M). There were 2 sets of solutions with normetanephrine, one for potassium induced and one for tyramine induced release of noradrenaline.

When preparing the uterine strips for this expt., care was taken to divide each pair of uterine horns into 4 equal pieces. The mean weight of four sets of strips from untreated and estrogenized uterus, respectively is shown in Table I. There was little difference between the weights of tissues within the groups of untreated and estrogeni reparations, whereas



Rate coefficient of ³H-noradrenaline efflux in isolated rat portal vein. Solid symbols: non-estrogenized. Open symbols: estrogen treated. Bathing solutions as indicated below the graph. Na = Na-free, K = K-Krebs. Values are means ± S.E. of 7 expts.

the values of A, Q and W obtained from Table I, the extraneuronal ³H-noradrenaline concentration was found to be 0.24 pmol/mg in the non-estrogenized and 0.25 pmol/mg in estrogen treated uterus. These concentrations were multiplied by weight and subtracted from the total amounts of ³H-noradrenaline in the tissue. The remaining amounts were considered as the neuronal ³H-noradrenaline, were 45.4 pmol in non-estrogenized and 38.3 pmol in estrogen treated preparations, respectively. The calculations also suggested that the amount of ³H-noradrenaline accumulated by the adrenergic nerves in the presence of desipramine, was larger in the estrogen treated (10.6 pmol) than in the non-estrogenized (2.3 pmol) uterine strips.

The total amount of noradrenaline in the control preparations (considered as representing the whole uterus) would be approximately 130 pmol according to the values previously found for the whole uterus including the cervix (Falck *et al.* 1974). Thus the estimated amounts of ³H-noradrenaline, accumulated in the adrenergic nerves under control conditions (see above), represented about 30 % of the endogenous transmitter.

Portal vein

The non-estrogenized and estrogen treated strips of the portal vein weighed 5.5 ± 0.4 mg and 14.2 ± 0.4 mg, respectively. The contents of ³H-noradrenaline in the strips immediately before they were exposed to K-Krebs were 2.49 ± 0.12 nmol/g in the non-estrogenized and 1.75 ± 0.12 nmol/g in the estrogen treated preparations. As the concentration of noradrenaline in the rat portal vein has been found to be 2.2 µg/g (Häggendal *et al.* 1970), the accumulated ³H-noradrenaline in the present experiments represented 20–25 % of the endogenous transmitter.

release The values were calculated by subtracting the efflux rate in the last fraction of Na Krebs from the rate of release during the subsequent 1 min immersion in K-Krebs or tyramine-containing Na Krebs. In both the control and the normetanephrine series the potassium induced release of ^{3}H noradrenaline was larger in non-estrogenized than in estrogen treated preparations. The desipramine treated strips released only small amounts of ^{3}H noradrenaline when immersed in K Krebs. Tyramine in Na-Krebs had a much weaker effect on ^{3}H-noradrenaline release than potassium this release was not inhibited by estrogen treatment.

It was also of interest to estimate the amount of ^{3}H-noradrenaline that was present in adrenergic nerves when release was triggered by potassium. Provided that the desipramine induced block of neuronal uptake of noradrenaline was effective, the concentration of extraneuronal ^{3}H-noradrenaline would be given by the tracer concentration found in the desipramine treated tissue. Accordingly the neuronal ^{3}H noradrenaline should be derived from the difference between the total amount of tracer in the control and the amount of extraneuronal ^{3}H-noradrenaline The nerve bound ^{3}H-noradrenaline estimated in this way was 43 1 pmol in the non-estrogenized and 27.2 pmol in the estrogen treated strips. These values suggest that estrogen treatment decreased the neuronal uptake under control conditions. However the possibility exists that estrogen reduces the blocking effect of desipramine on the neuronal uptake of noradrenaline. This would give an underestimation of the neuronal content of ^{3}H noradrenaline in the estrogenized preparations.

An attempt was therefore made to differentiate between neuronal and extraneuronal ^{3}H noradrenaline by calculations that were independent of a complete inhibition of neuronal uptake by desipramine. To perform these calculations the following assumptions were made 1) The rate of potassium induced ^{3}H noradrenaline efflux was proportional to the nerve content of tracer This assumption is partly based on the previous findings that thymoleptic drugs had either no effect (Paton 1973) or only a slight inhibitory action (Hägg-dal *et al* 1972) on ^{3}H noradrenaline efflux when used in concentrations similar to that used for desipramine in the present study 2) The concentration of extraneuronal ^{3}H-noradrenaline was equal in control and desipramine treated tissue. This is reasonable as the uterine contents of ^{3}H-noradrenaline were not affected by normetanephrine (see Table I), a more effective inhibitor of extraneuronal uptake than desipramine (Iversen 1967). 3) The weight of adrenergic nerves was negligible in relation to the weight of extraneuronal tissue in the uterus.

On the basis of these assumptions the following equation applies

$$\frac{A_c}{Q_c - B\ W_c} = \frac{A_d}{Q_d - B\ W_d}$$

A – rate of potassium induced ^{3}H-noradrenaline efflux
B – extraneuronal ^{3}H-noradrenaline concentration
c – index of control tissue
d – index of desipramine treated tissue
Q – ^{3}H-noradrenaline in whole tissue
W – tissue weight

r calcium by the addition of tyramine (Lindmar *et al.* 1967 García *et al.* 1976). The t study showed that the tyramine induced release of ³H-noradrenaline in estrogen d preparations is undiminished. This further supports the idea that the estrogen in- l inhibition of noradrenaline release is mediated through calcium dependent mechan- The finding that tyramine induced release of ³H-noradrenaline was unaffected by gen strongly suggests that the amount of H-noradrenaline, available for release in the terminals, is about the same in non-estrogenized and estrogen treated uterus. This is reement with the calculations shown under Results.

rat portal ein responded like the rat uterus with an increased release of ³H-nor aline hen immersed in the potassium-rich solution. This is consistent with a previous on potassium contractures in the rat portal vein, suggesting that released intramural drenaline exerts a contractile effect which adds to the contracture produced by potas- itself (Bengtsson 1977 a). The previous findings also suggested that the adrenergic nce in the rat portal vein was reduced by estrogen treatment, probably through a ction of noradrenaline release. This idea is supported by the present results, as estrogen ment significantly reduced the potassium induced release of H-noradrenaline. It is mable that the basic mechanism for this reduction is the same as that already discussed he uterus.

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The rate coefficient of ^{3}H-noradrenaline efflux decreased from an initial value of [illegible] 0.05/min to 0.02–0.03/min during the 23 min of washout in Na-Krebs (Fig. 4) and [illegible] larger in the non-estrogenized than in the estrogen treated preparations. This [illegible] remained when the portal veins were transferred from Na$^+$ to K$^+$ Krebs. The rate [illegible] of ^{3}H noradrenaline efflux increased to approximately 0.07/min during the first [illegible] potassium rich solution, after which the efflux rate showed a decline that [illegible] nounced in the estrogen treated than in the non-estrogenized portal veins. The [illegible] potassium on ^{3}H noradrenaline efflux was calculated as the area under the curve [illegible] in K$^+$ Krebs above the level of the rate coefficient in the last tube containing Na-Krebs. The values obtained in this way were 0.235 ± 0.13 in non-estrogenized and 0.1[illegible] estrogen treated portal veins ($p < 0.05$)

Discussion

Previous studies on potassium induced contractures in smooth muscle have shown [illegible] tractile effects that have been attributed to the release of intramural noradrenaline (Gr[illegible] 1966 Gibson and Pollock 1973 Bengtsson 1977 a) The present investigation has pro[illegible] direct evidence for this idea by showing a close correlation between increased ^{3}H-noradrena[illegible] line efflux and relaxation in the non-estrogenized rat uterus.

The initial high output of ^{3}H-noradrenaline in the isotonic potassium solution de[illegible] rapidly in spite of continued stimulation with potassium. A similar finding has been re[illegible] previously (Garcia *et al* 1976) it appears from that study that the decline in output [illegible] prolonged stimulation with potassium is due to failure of transmitter mobilization [illegible] than to exhaustion of releaseable transmitter stores.

The potassium induced release of ^{3}H-noradrenaline was considerably reduced [illegible] uterine strips treated with estrogen A smaller fraction of released ^{3}H-noradrenaline [illegible] appear in the bathing medium if estrogen induced a more effective uptake by the [illegible] neuronal tissue or a decreased diffusion rate through the extracellular space in the [illegible] The fact that neither tissue content of tracer nor potassium induced efflux were affected by normetanephrine indicates that the extraneuronal uptake was very small under [illegible] experimental conditions used Impaired diffusion is also an unlikely explanation of [illegible] estrogenic effect as the rate of noradrenaline flux into the uterine tissue is evidently not [illegible] fluenced by estrogen treatment. Thus depolarized uterine strips attain more than 9[illegible] maximum relaxation to a given dose of noradrenaline in 1 min, whether or not estro[illegible] treated (unpublished observations)

In view of these results, it is reasonable to suggest that the inhibition of ^{3}H-noradren[illegible] release is exerted through mechanisms regulating the release of transmitter from the [illegible] ergic nerve endings. Potassium induced release of noradrenaline is dependent on ca[illegible] in the extracellular medium (Kirpekar and Wakade 1968, Garcia *et al* 1976). Thus estr[illegible] might inhibit transmitter release by reducing the entry of calcium ions into the nerve term[illegible] In support of this idea the present results show that the ability of calcium to release [illegible] adrenaline disappears after estrogen treatment (see Fig. 1 2, 3)

It is of interest to note that noradrenaline release is produced in the absence of [illegible]

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Effects of pressure on fast axoplasmic flow An *in vitro* study in the vagus nerve of rabbits

By

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Abstract

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Using a radially directed stream of fluid it is possible to compress a section of a nerve in vitro by known pressures without interrupting the supply of oxygen and nutrients to the nerve. Motor neurons of the vagus nerve of rabbits labelled with ³H-leucine were used at room temperature When the nerve was subjected to mmHg pressure fast axoplasmic flow was not altered, but at 30 mmHg there was a slight but consistent block, which was even more marked at 60 mmHg and still more at 90 mmHg. The pressure induced block of axoplasmic flow was reversible when 60 mmHg pressure was applied for as long as 4 h, when the nerves were compressed for 15 h there was reversibility in only 5 out of 8 cases.

Elevated intraocular pressure blocks fast axoplasmic flow (FAF) at the level of the lamina cribrosa in the optic nerve. This has been demonstrated unequivocally for both the anterograde FAF coming from the eye (Hamsson 1973 Anderson and Hendrickson 1974) and the retrograde FAF—which enters the eye (Minckler *et al* 1977). It is not clear whether the pressure induced block of FAF in the optic nerve is mechanical or ischemic in origin. Since this is of crucial importance, it was decided to attack the problem using an in vitro system. The present study reports a technique for exerting pressure on the vagus nerve without interrupting the oxygen supply and presents some results using this technique.

Materials and methods

Animals Albino rabbits (New Zealand White) of either sex weighing between 2 0 and 3 5 kg were anaesthetized with 7 ml/kg of 25 % (w/v) urethane solution, administered intraperitoneally or slowly intravenously When surgical anaesthesia had been attained the animals were placed under a heating lamp to keep the body temperature around 37 C

Labelling of the motor neurons of the vagus nerve The method originally described by Miura (1963, see also Frizell 1974, for more detailed description) was used and is described here only briefly 30 µl ³H-leucine (4-4-5-³H leucine, specific activity was 60 C mmol, concentration 1 µCi/µl) was applied to the floor of the fourth ventricle in the vicinity of the superior nucleus of the vagus The application was done with a micro-

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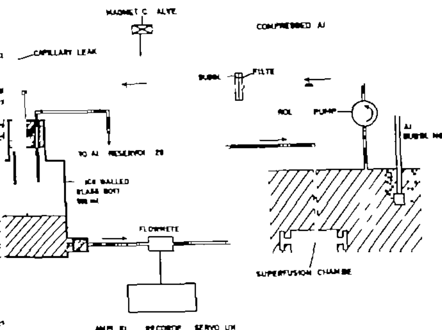
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Arrangement for superfusion of nerve with constant flow. For further explanation see text.

... diameter of 1.1 and 1.3 mm. The position of the hole in the probe was indicated by black paint and it was found that the hole was at the center of the gap when pressure was at its maximum. However the resolution was not sufficient to decide if this was true better than 0.1 mm. For reasons of symmetry it is assumed that the position of the gap is at the peak pressure, even if this is not proved.

Determination of radioactivity. The nerves were cut into 2–4 mm pieces and kept for 24 h in 10% trichloroacetic acid (TCA) at 4°C. Pilot studies showed that this treatment was sufficient to extract the TCA soluble fraction from the nerve tissue. The TCA soaked nerve pieces were either combusted in Packard 306 Tri... sample oxidizer or treated with Soluene® to which scintillation liquid was added when digestion was complete. The radioactivity was determined in liquid scintillation counter and corrected for quenching when necessary. The value for each nerve segment is expressed as percentage of the total amount in the whole nerve.

Results

Calibration of the superfusion chamber

The pressure distribution within the superfusion chamber is shown in Fig. 3. For clarity only two sets of conditions are shown. At given flow the gap size defines the shape of the curve. The curves were analysed with regard to the following three parameters: the maximum pressure value (in mmHg), the distance in mm between the 50% points on the limbs and the steepest slope of the limbs in mmHg/mm. In Fig. 4 the maximum pressures are plotted against different flows for different gap openings. It is apparent from this graph that the smaller the gap and the greater the flow rates, then the greater is the maximum pressure. Values between 30 and 150 mmHg could easily be achieved. While the slope also depends on the flow rate at ...

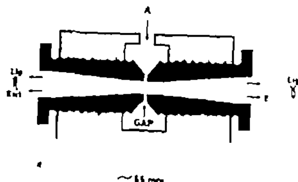


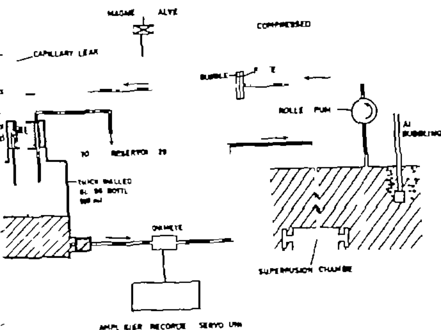
Fig. 1 Superfusion chamber. The two identical collinear screws, 27 mm long, are made of plexiglass. Their central channel has a diameter of 2.5 mm, is cylindrical in [illegible] and expands conically to 5 mm. The nerve is mounted within the channel. Fluid at a constant rate enters at A and runs radially through the gap, compresses the nerve and leaves the channel along the nerve (to the [illegible] nodosum).

syringe (Agla) at a rate of 1 2 μl/min. After 3.5 h the animal was killed by either an air embolus or a [illegible] i.v. injection of an overdose of urethane. Then, without delay 50–70 mm of both cervical vagi were [illegible] between two ligatures. The proximal ligature was applied just rostral to the ganglion nodosum. The [illegible] nerves were then immediately transferred into cold aerated superfusion solution and freed from connective tissue under the microscope. Care was taken not to touch or squeeze the nerve

The composition of the superfusion solution was. 1 000 ml 0.9 % NaCl, 40 ml 1 15 % KCl, 12.7 ml 1 [illegible] $CaCl_2$, 10 ml 2.11 % KH_2PO_4, 10 ml 3 13 % $MgCl_2 \cdot 6H_2O$, 210 ml 0 1 M $Na_2HPO_4 \cdot 2H_2O$, 2.57 g [illegible]

Localized compression of a nerve The method was designed so that a small segment of a nerve could be compressed by an applied constant pressure without interrupting the supply of oxygen. The device is shown in Fig. 1 The nerve is placed in the longitudinal channel of the chamber Aerated superfusion solution enters the chamber at A at a constant and rapid rate and flows centripetally through an adjustable gap where it impinges on the nerve and escapes axially along the nerve A positive pressure is thus applied in a radial direction around the whole perimeter of the nerve as it passes through the gap. The channel for the nerve runs through the long axis of two collinear plexiglass screws and the gap is formed between their ends. Gap size can be adjusted by turning the screws which can be locked by counternuts No attempt was made to calculate the complex fluid dynamics within the longitudinal channel. Instead the pressure distribution in the channel was measured by a probe (see below). The pressure profile was calibrated for different flow rates passing through the chamber for different gaps. Unfortunately it was technically impossible to record the pressure within the chamber continuously during an experiment. However by maintaining a constant flow at a given gap size the shape of the pressure distribution could be kept constant. Fig. 2 shows the whole arrangement. A roller pump (Watson-Marlow) sucks the superfusion solution and air from the surface of an aerated bath—thus keeping the surface level of the bath constant. The fluid passes through a filter (consisting of a nylon net with mesh aperture of 15 μm) and enters a thickwalled glass bottle from which the fluid is driven by air pressure and passes through a flowmeter into the superfusion chamber in the bath. The flowmeter consists of a constriction in a plexiglass tube and a differential pressure transducer (Sanborn, type 267 B). The flowmeter is part of a servo system maintaining constant flow If the flow decreases below the present value, a magnetic valve opens and air pressure is applied. To dampen out the pressure fluctuations the air space over the fluid in the bottle was connected to a 20 L expansion chamber. A very constant flow could thus be obtained for an extended period of time. Superfusion was usually maintained for 17 hrs at room temperature At the end of the experiment the nerve was cut across in the [illegible] center of the gap with a scalpel, the blade thickness of which fitted the gap. The two separated parts were then divided further into 2–4 mm pieces and the radioactivity determined as described below

Pressure probe The probe used for determining the pressure distribution in the channel for the superfusion chamber consisted of a steel tube 150 mm long and 0.9 mm in outer diameter A 0.1 mm hole was drilled approximately midway in the side wall. One end was plugged while the other was fitted to a plastic tube connected to a transducer (Sanborn, type 267 B). At the beginning of a measurement the probe, plastic tube and transducer were connected to a reservoir filled with gas-free water and situated about 100 cm above the transducer The probe was then introduced into the longitudinal channel. During this procedure the transducer and pressure probe were flushed continuously with gas-free water from the reservoir Flushing was discontinued when the probe was in place The superfusion chamber was then superfused at different flow rates and different gaps. At each particular setting the pressure probe was moved by means of a screw drive in steps of 0.1 mm along the channel and the pressure recorded. To check the effect of the probe itself on the pressure or pressure gradient within the channel, some measurements were also made with probes



2 Arrangement for superfusion of a nerve with constant flow For further explanation see text.

... diameter of 1.1 and 1.3 mm. The position of the hole in the probe was indicated by black paint and ... found that the hole was at the center of the gap when pressure was at its maximum. However, the resolution was not sufficient to decide if this was true to better than 0.1 mm. For reasons of symmetry it is assumed that the position of the gap is at the peak pressure, even if this is not proved.

Determination of radioactivity The nerves were cut into 2–4 mm pieces and kept for 24 h in 10% trichloro acetic acid (TCA) at 4°C. Pilot studies showed that this treatment was sufficient to extract the TCA soluble fraction from the nerve tissue. The TCA soaked nerve pieces were either combusted in a Packard 306 Tri-Carb sample oxidizer or treated with Soluene® to which scintillation liquid was added when digestion was complete. The radioactivity was determined in a liquid scintillation counter and corrected for quenching when necessary The value for each nerve segment is expressed as a percentage of the total amount in the whole nerve

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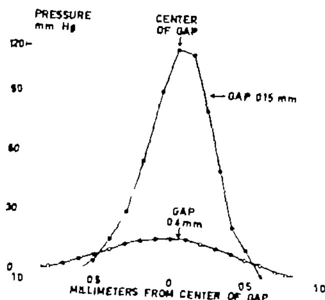


Fig. 3 Pressure distribution along the channel of the superfusion chamber. The rate of flow was 390 ml/min, gap 0.15 mm (●) and 0.4 mm (O).

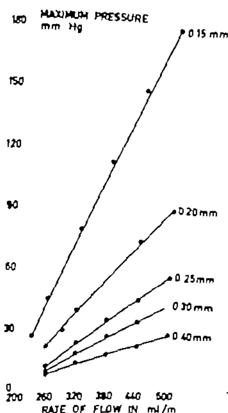


Fig. 4

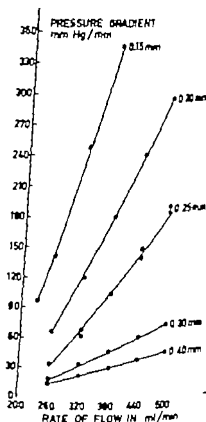
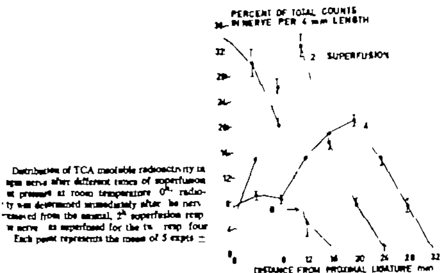


Fig. 5

Fig. 4 Maximum pressure when superfusion chamber is perfused with different rates of flow at different gaps.

Fig. 5 Steepest slopes of the pressure gradients, when the superfusion chamber is perfused with different rates of flow and different gaps



Distribution of TCA insoluble radioactivity in ... nerve after different times of superfusion at pressure at room temperature. 0[h] radio... ty was determined immediately after the nerv... removed from the animal; 2[h] superfusion resp. ... nerve ... superfused for the tw... resp. four ... Each point represents the mean of 5 expts ±

...en gap (Fig. 5) the width is independent of the flow rate and defined by the gap size. The ... diameter of the pressure probe did not change the results.

...he present paper reports result obtained using 0.2 mm and occasionally 0.4 mm gaps.

...rate of transport of tritium labelled TAC insoluble material

...umber of experiments were made without compression of the nerve. The distribution of ...oactivity along the nerve was determined either immediately after its removal (time 0) ...ther 2, 4 and 20 h. I... Fig. 6 the portions of incorporated ³H labelled material at 2 and 4 h ...er removal of the nerves are shown. The crest of the 4 h superfused nerve is about 8 mm in ...nt of the "two hour" crest. Thus, the rate of transport was about 96 mm/24 h. When the ...rv... was kept for 20 h in a superfusion bath at room temperature the radioactivity was ...ated mainly at the distal ligature with only a small fraction at the proximal ligature. (The ...ure is not shown since the results are almost identical to Fig. 7 a.)

...ffect of pressures on fast axoplasmic flow

...hen a nerve section was subjected to 20 mmHg compression, the movement of labelled ...aterial was unaffected (Fig. 7 a). The 20 mmHg compression was achieved either by per...sion of the chamber with a flow of 250 ml/min and a gap of 0.2 mm, or in 2 out of 5 cases ...th a gap of 0.4 mm and ... flow of 375 ml/min.

...When the pressure was increased to 30 mmHg (290 ml/min, gap 0.2 mm) there was ... slight ...ut consistent hindrance of flow within the pressure area (Fig. 7 b). However, most of the ...roteins passed the pressure barrier and collected at the distal ligature.

...With 60 mmHg (400 ml/min, gap 0.2 mm) ... considerable fraction of proteins were arrested ...thin the pressure area. Most of the radioactivity, however, passed the barrier (Fig. 7 c).

When the pressure was raised to 90 mmHg (500 ml/min, gap 0.2 mm) most of the radio-

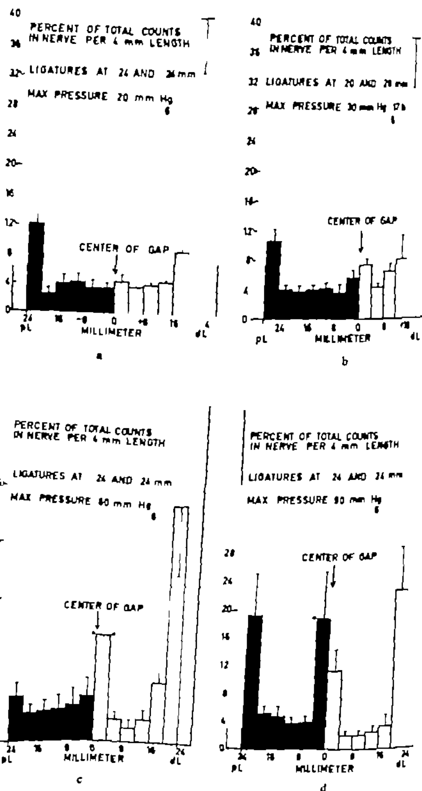
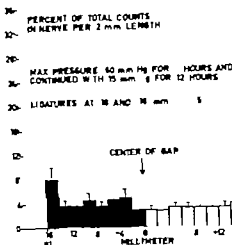


Fig. 7 a, b c, d Distribution of TCA insol ble radroactivity in the vagus nerve 17 h fter superfus room temperature. Direction of terograde axoplasmic flow Error indicat r = 1 S.E. pL and dL repr the positions of the proximal and distal ligatures resp Asterisks indicat significant difference betwee columns (paired t test, p 0.05)



8 Reversibility. Distribution of TCA ble radioactivity in the agus nerve 16 h uperfusion at room temperature. Direc of anterograde axoplasmic flow from left ght. pL proximal ligature, dL distal ure. Error indicator = S.E.

ty was blocked at the pressure area, although some passed the barrier and collected at distal ligature (Fig. 7 d).

is interesting to note that arrested radioactivity in the pressure area was found on both s of the pressure barrier. At 30 mmHg and 60 mmHg in 8 out of 11 cases the accumula- t even as more pronounced distal to the pressure barrier while at 90 mmHg this was erved (5 out of 6 cases).

he accumulation at the proximal ligature was about 10% of the total amount except in 90 mmHg experiments where it reached nearly 20%.

ersibility of the pressure induced block of FAF

ssure (60 mmHg) on a nerve section was released 17 hrs after the start, the nerve taken t of the chamber and two new ligatures applied 6 mm proximal and distal to the centre of pressure area. The nerves were then incubated in tissue culture medium TC 199 at 38°C 5 h using the procedure of McLean *et al.* (1976). In 5 out of 8 cases no excess radioac ity as found in the pressure area. However in three cases there was accumulation in- cating that the pressure induced FAF block had not been fully reversed.

When pressure (60 mmHg) was pplied for 4 hrs and the nerve then superfused with a flow ving less than 15 mmHg, there was no accumulation except at the distal and the proximal gatures (Fig. 8).

Discussion

his classical studies of axoplasmic flow Weiss (Weiss and Hallowell 1943) used arterial leeves to compress a nerve others have utilized spring clips (Denny-Brown and Brenner 944), tourniquets (Denny-Brown and Brenner 1955 Ochoa *et al.* 1971) or rubber membranes McLean and Sjostrand 1977). Although some of these studies were not intended to in- vestigate axoplasmic flow the methods have one main feature in common, effects seen may

be due to ischemia or asphyxia rather than to real mechanical obstruction. With the method presented here it is feasible to subject a nerve to adjustable, stable pressures. The principle of constricting a nerve by a flowing solution was first introduced by Bisby (1974) and the procedure used here is a further development of his method.

The fact that the pressure within the chamber cannot be recorded continuously throughout an experiment without interfering seriously with the hydrodynamics, is a disadvantage. But there was a high correlation between flow and pressure when measured with a pressure probe. This was tested repeatedly for up to 24 h, which was longer than the experimental times. There is no obvious reason why the flow/pressure relation should change considerably when the nerve was substituted for the probe

It is not known how much of the pressure applied to the surface is transmitted to the center of the nerve. No attempt was made to record the actual pressures within the nerve. If there was an appreciable pressure gradient from the outer to the inner layers due to rapid of the tissue, the effective pressure would be overestimated (or the degree of block underestimated)

The calculated rate of transport of FAF at room temperature (approximately 22 C) is about a third of the velocity reported by McLean *et al* (1976) in vagus nerves incubated at 38 C Axoplasmic flow is temperature sensitive with a Q of 2-2.3 (Ochs 1971). The rates of flow are thus in fairly good agreement

The distribution profiles after 20 h incubation at 22°C in the present expts. and incubation at 38 C (data from McLean *et al* 1976) are different At room temperature there was almost twice as much accumulated at the distal ligature than in the nerves incubated at the higher temperature at the proximal ligature this relation was reversed. This is most likely due to the temperature dependence of retrograde flow At both temperatures, the main part of the label would reach the distal ligature But only a small fraction of the retrogradely migrating proteins reached the proximal ligature at room temperature, *i.e* only those which were reversed en route while at 38°C there was enough time for a considerable redistribution to the distal ligature.

The present investigation has shown that axoplasmic flow is highly sensitive to a pressure step As expected, FAF inhibition is due to a pressure difference, not to the pressure per se Ochs (1974) found no inhibition of FAF when nerves were subjected to up to 5 atm in compressed air It is striking that already at a pressure of 30 mmHg some of the FAF was blocked. No block was discernible at 20 mmHg but since there is a noise level in the method even this low pressure could conceivably have had an effect The block increased with increasing pressure It cannot be decided if the effect observed in the whole nerve reflects the effect on the single axon It seems quite possible that an all or none effect exists. That some axons are more resistant than others is supported by findings of Quigley and Anderson (1976) in FAF block in the optic nerve of owl monkeys.

The effect of pressure steps was reversible at least up to 4 h. It is not yet understood why some nerves did not show reversibility after 15 h pressure This problem is of obvious importance in connection with reversibility of field changes in glaucoma.

Radioactivity accumulated on both sides of the pressure barrier though it was differently distributed at different pressures. At 30 and 60 but not at 90 mmHg the heaviest accumulation

was found immediately distal to the pressure barrier both when the results were pooled, and measurements from the single nerve, although not in all cases. Could this be due to a methodological error? It is assumed that the maximal pressure on the nerve is exerted at the of the gap, although this could not be proved. Could the maximal pressure may have out of centre, due to slight differences in the shape of the screws. However the pair was randomly selected out of six 'identical' screws for each new suprefusion and body of the chamber was turned right or left by chance. A methodological error seems ly and the observation must thus be regarded as a characteristic feature of axoplasmic

en the pressure was 90 mmHg, axoplasmic flow was strongly inhibited immediately mal to the pressure barrier and only relatively little protein was able to pass the pressure nd collect distally to the pressure barrier. It is interesting to note that the accumulation proximal ligature was somewhat higher in the 90 mmHg experiment than in those at 30 0 mmHg. It is a well known feature of FAF that components reaching a ligature are ted and move in retrograde direction (Frizell 1974). The finding at 90 mmHg pressure d suggest that some protein was reflected at the pressure barrier and moved retro-ly towards the proximal ligature.

e finding that the accumulation of protein is more pronounced distal to pressure barrier or 60 mmHg may be due to accumulation of retrogradely migrating proteins, which had d the pressure barrier in the anterograde direction and then became reflected en route now accumulated distally to the pressure barrier. However the phenomenon is not yet ly understood.

chronic simple glaucoma 30 mmHg intraocular pressure can be deleterious to the optic e. The present study shows that pressure in this range is already able to affect fast axo-mic flow albeit in peripheral nerve and at room temperature.

superfusion chamber set-up including the electronics was constructed by Folk Hogberg and Claes-Ivar ... This ... gratefully acknowledged. The author also thanks Maria Carlsson for excellent technical ... The investigation was supported by grant EYE 00231 13 from the National Eye Institute U.S. ... to Professor E. Bárány.

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Vasopressin release induced by hemorrhage in the goat

By

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Abstract

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of moderate hemorrhage were studied in normovolemic and hydrated conscious goats. Plasma vasopressin (AVP) in jugular vein blood did not rise in response to bleeding of 8 and 12 ml/kg After blood loss of 16 ml/kg an abrupt and conspicuous rise in plasma AVP (to > 10 times basic was seen in 50 % of the experiments, whereas no rise in AVP occurred in the remaining experiments. ing at 20 and 24 ml/kg gave more consistent AVP response. Hydration did apparently not reduce AVP-response to bleeding. The plasma vasopressin concentration returned to pre-hemorrhage about 3 h after bleeding, i before the blood was re-transfused. A minor and inconsistent rise in renin activity occurred in response to bleeding Central venous pressure fell during hemorrhage and stayed depressed until the blood was re-transfused. Since the depression was of the same order in all experiments, and was still present when plasma AVP had returned to pre-hemorrhage level, the fall in pressure was obviously not the main cause of the AVP-response. However the pronounced rise plasma vasopressin was correlated to fall in the arterial pressure and to hyperventilation. No urge to developed during any of the hemorrhage experiments. It is concluded that the goat does not readily respond to hemorrhage with vasopressin liberation. When it happens, pressor amounts of the hormone released, and fall in arterial pressure and/or increased stimulation of respiratory chemoreceptors appear(s) to be the ultimate cause(s) of the release. This thirst-independent regulation of vasopressin secretion is obviously of no importance in day to day control of water balance but may help t maintain the arterial blood pressure in emergency situations.

words hemorrhage, vasopressin, thirst, plasma renin activity arterial blood pressure, central venous pressure, goat

The release of vasopressin (antidiuretic hormone) from the neurohypophysis is influenced as well by the composition as by the volume of the extracellular fluid (ECF) Secretion of the hormone in response to normal, or elevated ECF [Na] (and osmolality) is apparently governed by sodium sensitive receptors which predominantly are located near the anterior portion of the third cerebral ventricle (*cf* Andersson 1977). The volume-dependent regulation, on the other hand, by all probability consists of both reflex and humoral links. Impulses from cardiovascular distention receptors and baroreceptors appear to exert a tonic inhibitory

influence upon the neurohypophyseal liberation of the hormone (cf. Gauer Henry ... Behn 1970), whereas hypovolemia-induced renal renin release, with subsequent formation of angiotensin II, may stimulate vasopressin secretion (cf. Severs and Daniels-Severs ... Such an effect of angiotensin II appears to be mediated by the same cerebral receptors which are affected by the extracellular [Na⁺] (cf. Andersson 1977).

In support of the idea that juxtaventricular sodium sensitive receptors play an important role in the regulation of vasopressin secretion is the observation that reduction of the cerebrospinal fluid (CSF) [Na⁺] (induced by intracerebroventricular administration of ... lyte solutions) elicits a water diuresis in non-hydrated normovolemic goats (Eriksson ... Accordingly, prolonged (3 h) infusions of glycerol solution into the lateral ventricle ... were recently found to cause a sustained depression of the plasma vasopressin level ... persistent water diuresis (Olsson *et al.* 1978). Since the water diuresis was not compensated for by drinking, it gradually induced pronounced hypernatremia and hypovolemia. However, towards the end of the 3 h glycerol infusions the plasma vasopressin started to ... again in spite of the fact that CSF [Na⁺] remained subnormal. Since this "escape" ... have been a manifestation of volume-dependent regulation of vasopressin secretion, it became of interest to study to what extent hemorrhage stimulates vasopressin release, renin liberation, and thirst in the goat.

Methods

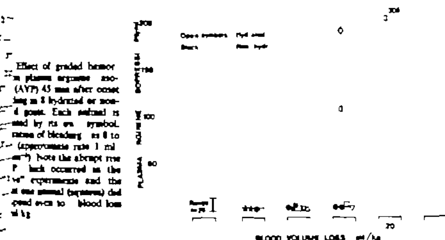
Animals. 8 horned female goats (b.wt. 35–55 kg) were used in a total of 26 expts. The animals were ... confined in metabolism cages where they had free access to water and were fed hay 3 times a day ... afternoon the goats received 300 g of commercial grain mixed with 6 g of NaCl added.

Surgery. In 4 of the goats a permanent polyvinyl catheter was inserted into the carotid artery via the superficial temporal artery under general anesthesia (mebumal). To avoid clotting, the catheters were flushed once daily with isotonic saline and were then filled with heparin solution (1 000 IE/ml).

Experimental procedure. In the morning before an experiment the animals were allowed to feed and drink *ad lib.* until about 10 o'clock. It was then placed in a modified Pavlov stand which prevented it from lying down. Downwards head movements were largely prevented by connecting (via a leather strap) the horns to a bar above the head. However, the goat could still move its head rather freely from side to side. The animal was not allowed to eat when placed in the Pavlov stand but was offered water every hour. A cannula (to be used for bleeding and the taking of blood samples) was inserted into one jugular vein. In 12 expts. a polyethylene catheter for recording of central venous pressure was inserted via the contralateral jugular vein to a level about 5 cm in front of the right atrium. After control blood samples had been taken for analyses, the goats were bled 8, 12, 16, 20 and 24 ml/kg at a rate of approximately 1 ml kg⁻¹ min⁻¹. The blood was collected in heparinized glass bottle and was re-transfused to the goat towards the end of the experimental period. In 14 of the experiments the goats were hydrated. They had then been given 100 ml/kg of 38°C water by stomach tube into the rumen about 90 min before bleeding started.

Recordings of blood pressure and respiratory rate. The carotid blood pressure was measured in four of the animals via the permanently implanted catheter which was then connected to a Statham transducer. The systolic/diastolic pressures were recorded on a polygraph. Central venous pressure was recorded in the same manner via the jugular vein catheter. When not registered during recordings of carotid blood pressure, the heart rate was determined at intervals by auscultation. The respiratory rate was simply determined by counting the expirations.

Blood and urine sampling. Blood for hormone radioimmunoassays was collected via the jugular cannula and was drained into ice-chilled centrifuge tubes with 0.3 M Na EDTA as anticoagulant. The blood was immediately centrifuged at +4°C whereupon the plasma was stored at −20 C until radioimmunoassays were performed. Samples for determinations of hematocrit, (Ht) and Plasma [Na⁺], [K⁺] and osmolality were collected in heparinized tubes, whereas blood for serum protein determination was taken in ...



... Effect of graded hemor... on plasma arginine vaso... (AVP) 45 min after onset ... ing in 8 hydrated or non-... d goats. Each animal is ...ated by its own symbol. ...rates of bleeding was 8 to ... (approximate rate 1 ml ...min⁻¹). Note the abrupt rise ... which occurred in the ...ed experiments and the ... at one animal (squares) did ...pond even to blood loss ... ml/kg

...without anticoagulants. Urine was collected in 30 min samples via a Foley catheter inserted into the ...y bladder.

...ical techniques. Determinations for plasma arginine vasopressin (AVP) were made according to ...d previously described by Fyhrquist *et al.* (1976 b). The sensitivity of this method is 0.2 pg/ml. Radio...assays of plasma renin activity (PRA) were made according to Fyhrquist *et al.* (1976 a). Plasma ...one [Na⁺] and [K⁺] were measured by internal standard flame photometry using an IL 343 flame ...meter. An Advanced Instruments Inc. osmometer was used for determinations of the osmolality ...ne fluids. The H... values were obtained by centrifugating (in triplicates) microhematocrit tubes at ...0 rpm for 5 min. Serum protein was determined by the Biuret method. Values including ± figures rep...means and S.E. Student t-test was employed for the statistical analyses.

Results

...opressin release and plasma renin activity

... AVP concentrations observed in jugular vein plasma 45 min after the start of the 8 to ...min bleeding periods are shown in Fig. 1. Five of the goats were bled 8 and/or 12 ml/kg ...10). On no occasion did this moderate blood loss induce any noticeable elevation of the ...ma vasopressin level. In subsequent experiments the same 5 animals were bled 16 and/or ...ml/kg (n = 11). The effect on plasma AVP was either conspicuous (range 30 to 102 pg/ml, ...5) or negligible (range 2 to 9 pg/ml, ... 6). In one of the goats even the blood loss of ...ml/kg was ineffective in raising the AVP. Another animal was bled also 24 ml/kg. The ...ect on plasma AVP was then not much greater than that obtained at 20 ml/kg and in ... of the 16 ml/kg expts. (50 as compared to 30 and 47 pg/ml).

Three goats, bled *de novo* at 16 ml/kg (n = 3), all responded with large rises in the plasma ...sopressin concentration (60 to 191 pg/ml). When a greater blood loss was induced in one ... these animals (20 ml/kg) the AVP rose to 305 pg/ml. The results presented in Fig. 1 gives ...e general impression that hemorrhage did not cause any graded vasopressin response in ...gular vein plasma, but rather an abrupt release of considerable amounts of the hormone ...hen a certain threshold of blood loss had been reached. It is also evident that there were ...marked interindividual differences in the sensitivity to bleeding, and that the amount of

TABLE I Hemorrhage induced effects on jugular plasma arginine vasopressin (AVP) (pg/ml). [illegible] (about 1 ml kg^{-1} min^{-1}) was started at zero time and lasted for 8 to 26 min. N–R = [illegible] in which no noticeable rise in AVP occurred. R = AVP-positive experiments. Duration of transfusion 20–35 min.

Time	Hemorrhage				
	8 and 12 ml/kg	16 ml/kg		20 and 24 ml/kg	
	N–R (n = 10)	N–R (n = 5)	R (n = 5)	N–R (n = 1)	R (n = 5)
–45	2.6±0.4	4.1±0.5	6.1±1.1	5.9	4.3±2.8
15	2.5±0.4	4.1±0.5	6.4±2.2	4.6	4.1±1.2
	Bleeding				
45	2.8±0.3	3.6±0.6	97.4±25.4	8.9	115.8±48.9
75	2.3±0.4	2.7±0.8	46.1±19.1	8.9	61.8±12.0
105	2.0±0.3	3.3±0.5	30.7±15.9	5.8	39.0±11.1
135	—	—	16.6±6.7	—	14.9±5.9
165	—	—	14.0±5.8	—	4.9 (n = 3)
195	—	—	7.0±1.9	—	2.4 (n = 3)
225	—	—	7.6±2.1	—	3.5 (n = 2)
	Re-transfusion				
5 min after retransf	2.2±0.6	3.9±0.5	7.7±2.7	5.0	8.2±2.5

vasopressin released was not clearly related to the volume of blood loss. Furthermore hydration was not seen to diminish the hormone release in response to hemorrhage.

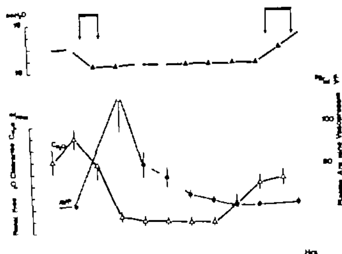
The plasma AVP was determined at 30 min intervals after the first post-hemorrhagic sample had been taken (45 min after onset of bleeding). The results are presented in Table I. Here the "AVP positive" experiments have been separated from those in which no noticeable rise in AVP occurred. In the positive experiments the peak level of plasma AVP [illegible] always found in the first post-hemorrhagic sample. Plasma AVP then gradually returned [illegible] pre-bleeding values. This occurred within 3 h after bleeding, *i.e.* before the lost [illegible] volume was transfused back into the animals.

As expected, "AVP-positive" hemorrhage always induced a pronounced and [illegible] inhibition of the water diuresis of hydrated goats. As shown in Fig. 2 this inhibition of the [illegible] water diuresis was well correlated in time with the rise in AVP. Thus, after having [illegible] negative for about 2 h, the renal free water clearance returned to positive values before [illegible] transfusion of the blood. In contrast, the course of the water diuresis never became affected [illegible] when hemorrhage in hydrated goats did not cause measurable increase in plasma AVP.

In most experiments the bleeding caused some increase in PRA (Table II).

Absence of thirst

In none of the 26 hemorrhage expts. did the goats drink the water offered to them at regular intervals. That the animals were restrained to a certain extent does not seem to be the [illegible] reason for the absence of drinking motivation. Thus, on other occasions two of the goats [illegible] were deprived of water for 24 h. They were then placed in the Pavlov stand where they were [illegible] restrained in the usual manner. When offered water both goats immediately drank about 2 l of water



2. Effects of bleeding (B) (blood loss 16 or 20 ml/kg b wt.) on central venous pressure (CVP), plasma vasopressin (AVP) and renal free water clearance (C_{H_2O}) in 6 AVP-positive experiments performed in 4 hydrated goats (compare Fig. 1). Note that the CVP remains depressed until the lost blood was re-transfused (R), whereas plasma AVP returns to pre-hemorrhage level, and the C_{H_2O} to positive value, 40 min earlier. The rate of blood loss was about 1 ml kg^{-1} min. Vertical bars: S.E.

Circulatory and respiratory effects

The carotid blood pressure was followed during 10 expts. (4 animals). Hemorrhage causing blood loss of 8 and 12 ml/kg (4 recordings, 3 goats) did not induce any fall in the mean arterial pressure. However, a slight decrease in pulse pressure and about 50% increase in heart rate occurred towards the end of the bleeding periods. Neither did bleeding at 16 and 24 ml/kg cause any fall in the arterial pressure of the goats which did not respond with vasopressin release. In two goats responding with pronounced elevation of plasma AVP to hemorrhage of 16, 20 or 24 ml/kg, a 15 to 25 mmHg fall in mean carotid pressure was seen at the end of bleeding. The drop in mean pressure was accompanied by a marked decrease in pulse pressure and a rise in respiratory frequency from about 15 to about 200/min. The arterial blood pressure and the respiratory frequency did not return to pre-hemorrhage values until about 2 h later. Polypnea of the same intensity and duration also developed in all other experiments in which a rise in plasma AVP was seen. However, no increase in respiratory frequency occurred in the AVP-negative expts.

The central venous pressure (CVP) was followed during 12 expts. (bleeding at 8 and 12 ml/kg, n = 4 at 16 ml/kg, n = 7 and at 20 ml/kg, n = 1). Bleeding invariable induced depression of CVP which was of the same order in all expts. (about 6 cm H_2O) regardless of whether an AVP-response was obtained or not. The CVP remained depressed until the lost blood volume was transfused back into the goats. Then the CVP rose above pre-bleeding level (Fig. 2).

Blood composition

There were no noticeable changes in either plasma [Na], [K] or osmolality during the course of any of the expts. Serum protein had fallen slightly (about 5%) 105 min after the

TABLE II. Hemorrhage-induced effects on jugular plasma renin activity (PRA) (ng ml⁻¹ h⁻¹) Bleeding (about 1 ml kg⁻¹ min⁻¹) was started at zero time and lasted for 8 to 26 min N-R experiments in which no noticeable rise in AVP occurred. R = AVP-positive experiments. Duration of transfusion 20–35 min.

Time	Hemorrhage				
	8 and 12 ml/kg N–R (n = 10)	16 ml/kg N–R (n = 5)	R (n = 5)	20 and 24 ml/kg N–R (n = 1)	R (n 5)
−45	0.26 ± 0.05	0.21 ± 0.15	0.75 ± 0.31	0.26	0.74 ± 0.21
−15	0.26 ± 0.07	0.34 ± 0.12	0.65 ± 0.30	0.32	0.60 + 0.12
	Bleeding				
45	0.48 ± 0.09	0.39 ± 0.18	0.81 ± 0.40	0.32	0.69 ± 0.33
75	0.57 ± 0.13	0.39 ± 0.16	0.88 ± 0.37	0.66	0.53 ± 0.17
105	0.53 ± 0.18	0.60 ± 0.39	1.29 ± 0.51	0.24	0.86 + 0.27
135	—	—	1.69 ± 0.87	—	0.67 + 0.17
165	—	—	2.11 ± 1.20	—	1.17 (n 3)
195	—	—	1.95 ± 0.95	—	1.19 (n 3)
225	—	—	2.02 ± 0.78	—	1.26 (n 2)
	Re transfusion				
5 min after retransf	0.36 ± 0.12	0.39 ± 0.25	1.25 ± 0.65	0.18	0.63 + 0.17

more moderate bleeding (8 and 12 ml/kg) This fall was not statistically significant. However a significant ($p < 0.01$) drop in serum protein was recorded 105 min after start bleeding when the goats were bled 16 and 20 ml/kg (from 6.7 ± 0.1 to 5.8 ± 0.2 g/100 ml). The reduction in serum protein was of the same order in "AVP-positive" and "AVP-negative" expts. However a difference in the effects on Ht was observed between these two groups of expts. A 14% reduction in Ht ($p < 0.05$) had occurred 105 min after the onset of bleeding (16 and 20 ml/kg) in the "AVP-positive" expts, whereas the fall in Ht was only 5% when bleeding at these degrees did not induce a rise in AVP The 8 and 12 ml/kg blood loss caused even less reduction in Ht (about 3%)

Renal salt excretion

No apparent change in renal potassium excretion occurred during any of the expts. However a decrease in renal sodium excretion was observed in response to the blood loss of 16, 20 and 24 ml/kg in the "AVP-positive" expts. Here the Na excretion gradually dropped from 54 ± 13 μmol/min to 15 ± 6 μmol/min in the third post-bleeding period. It remained at this low level until the blood was retransfused. The corresponding values in the "AVP-negative" expts. were 54 ± 18 μmol/min (before bleeding) and 59 ± 28 μmol/min.

Discussion

Already 40 years ago Rydin and Verney (1938) showed that hemorrhage, accompanied by a transient fall in arterial blood pressure, causes an inhibition of the water diuresis in hydrated, conscious dogs. Since the inhibition was of much longer duration than the depression of the blood pressure, they concluded that it was caused by some humoral factor probably antidiuretic hormone, and not by changes in renal hemodynamics. studies have con

'ed that antidiuresis, developing in response to bleeding, in fact is due to release of pressin (cf Share 1969), but the mechanism behind hemorrhage-induced release of the mone remains a matter of controversy. Experiments in animals and man have provided ative evidence that cardiovascular distention receptors in the low pressure system are lved in the regulation of vasopressin secretion (cf Gauer et al 1970). However the ortance of such a mechanism in the control of water balance has also been called in tion (Goetz, Bond and Bloxham 1975). Studies in rats (Dunn et al. 1973), monkeys mold et al. 1977) and man (Goetz, Bond and Smith 1974), performed since radio-noassays of plasma AVP became possible, imply that substantial release of vaso-sin in response to hemorrhage does not occur until blood has been lost to such an extent the arterial blood pressure begins to fall. The results of the present study suggest that also holds true in the goat. Furthermore, the experiments also imply that the observed P response to bleeding was not due to reduced inhibitory inflow from distension receptors the low pressure side of the cardiovascular system. In the experiments where the arterial sure was recorded, a rise in plasma AVP was only observed when bleeding temporarily used hypotension, and the blood pressure never fell when recorded in "AVP-negative" morrhage experiments. The CVP on the other hand, was reduced to the same extent as l in "AVP-negative" as in "AVP-positive" expts., and remained depressed even when plasma AVP had returned to pre-hemorrhage concentrations (Fig. 2). It could be argued t the CVP was recorded in the anterior caval vein and not in the left atrium, which is sidered the principal location of distention receptors involved in the regulation of vaso-sin secretion. However it has been demonstrated (Henry, Gauer and Sieker 1956) t, within changes of 30% of the blood volume, the pressure in the large thoracic veins s and falls in parallel with the atrial pressures. That the observed conspicuous elevation plasma AVP should have been due to reduced activity of distention receptors in the pressure system is unlikely also for another reason. The rise in plasma AVP had more less the character of an "all or none" response (Fig. 1) with no clear-cut relation to the solute volume of blood loss. A much more graded AVP response would have been ex-cted if the vasopressin release had been a manifestation of reduced blood volume in the pressure system. It is likely that blood obtained from the jugular vein shows higher solute vasopressin concentration than that seen in peripheral blood. Thus, even minor ses in AVP-concentration ought to have been detected in this study.

Even though a fall in arterial pressure apparently had to occur before hemorrhage induced substantial vasopressin release, this must not a priori mean that reduced baroreceptor inflow was the crucial factor. In the present experiments the AVP response invariably was ac-companied by pronounced hyperventilation. No measurements of arterial pO_2, pCO_2 or pH were made, but it appears most likely that the hyperventilation was mainly a manifesta-tion of hypoxia. Forsling and Ullman (1976) have shown in anesthetized dogs that hypoxia causes increased vasopressin secretion, presumably by an effect on receptors at the medul-lary level. Therefore, it appears likely that diminished O_2-transporting capacity of the blood after bleeding might have contributed substantially to the large rises in plasma AVP seen in many of the present expts. Of interest in regard to the possibility that hypoxia may have caused or contributed to the hemorrhage-induced elevation of plasma AVP is also the

differential effects on hematocrit. In the "AVP-positive" expts. bleeding caused a considerably greater fall in hematocrit than did the corresponding blood loss in "AVP-negative" expts. Possibly the AVP-sensitivity to hemorrhage was negatively correlated to the amount of stored erythrocytes available for compensation of the blood loss. However, the greater fall of Ht in the "AVP positive" expts. may also have been a manifestation of hormone induced water retention.

The hypovolemia induced in the present animals caused only a minor rise in PRA. It appears most unlikely that this weak and inconsistent activation of the renin-angiotensin system (Table II) could have contributed significantly to the conspicuous vasopressin release seen in the "AVP-positive" expts. Thus, the vasopressin release which is obtained in response to infusions of rather unphysiological amounts of angiotensin II into the 3rd cerebral ventricle of the goat is apparently of much smaller order than that observed in the present study (Lishajko and Andersson 1975). Furthermore, since angiotensin is a potent dipsogen (*cf* Severs and Summy Long 1975) thirst ought to have developed in the "AVP positive" expts. if activation of the renin-angiotensin system had been the ultimate cause of the pronounced vasopressin effect The fact that no urge to drink developed in response to bleeding (sometimes followed by an almost 100-fold elevation of jugular plasma AVP; Fig. 1) shows that an emergency regulation of vasopressin secretion exists, which is largely independent of the regulation of water intake. It is in agreement with previous studies [illegible] other species which have shown that thirst is by far no consistent effect of rather substantial blood loss (*cf* Fitzsimons 1972). The purpose of this, apparently thirst-independent, release of huge amounts of AVP could be to assist in the maintenance of the arterial blood pressure in emergency situations. As previously pointed out (Goetz *et al* 1974) that mechanism is obviously of no importance in day to day control of water balance. The homeostatic control of water balance demands a much more subtle regulation of vasopressin secretion which is intimately linked to the regulation of water intake. This has recently been illustrated in the goat by measurements of plasma AVP during developing dehydration (Olsson *et al.* 1978). During two days of water deprivation the plasma AVP gradually rose concomitant with elevation of plasma [Na] However the AVP level in jugular vein plasma at the end of the dehydration period was only about 30% of that observed here in the "AVP-positive" expts. (Table I) This in spite of the fact that the two days of water deprivation had caused a [illegible] loss of body fluid which was several times that induced by the present hemorrhage.

Taken together the results presented here and the earlier vasopressin-data obtained in dehydration experiments (Olsson *et al* 1978) would indicate a dual role for vasopressin: one of importance in water economy and the other with the purpose of maintaining blood pressure in sudden hypovolemic hypotension

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Simultaneous measurements of capillary diffusion and filtration exchange during shifts in filtration-absorption and at graded alterations in the capillary permeability surface area product (PS)

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Abstract

RIPPE, B A KAMIYA and B FOLKOW *Simultaneous measurements of capillary diffusion and filtration exchange during shifts in filtration-absorbtion and at graded alterations in the capillary permeability surface area product (PS)* Acta physiol. scand. 1978 104 318–

The diffusion exchange of Cr EDTA, using the single injection indicator diffusion method, was ... simultaneously with estimations of the capillary filtration capacity (CFC) in an "isogravimetric" ... quarter preparation during artificial perfusion and maximal dilatation. Measurements were perform... constant flow and during 1) shifts in filtration-absorbtion, 2) alterations of perfused capillary ... (graded rarification of capillary network by microsphere injection) and 3) during alterations of perme... (i.e. infusion of histamine). At maximal vasodilatation CFC was 0.037 ± 0.001 ml/min mmHg 100 g ... PS for Cr EDTA 5.67 ± 0.13 ml/min 100 g. During filtration or absorbtion, Cr-EDTA transfer ... vessels to interstitium changed only slightly but the situation may well be different for solute transfer ... interstitium to vessels. Alterations in capillary wall area resulted in proportional changes in PS for ... EDTA while the CFC changes were always relatively smaller. Histamine increased CFC some ... with a marked increase in protein transfer while PS for Cr EDTA increased only marginally. The ... mine effect could be ascribed mainly to an increase in the number of large pores which, because of ... relative paucity are of little importance for small molecular diffusion exchange but highly importa... convective and macromolecular exchange.

Concerning capillary diffusion and filtration exchange, only few attempts have been ... to measure simultaneously and quantitatively both these processes under well contro... conditions and with independent techniques. Since transcapillary exchange of water-solubl... substances is considered to take place not only by pore-bound diffusion but also to a variabl... extent by convection, the sparsity of such studies may seem surprising which, however, ... a great extent is due to the considerable technical difficulties involved. In general it is accepted that the rapid transcapillary passage of lipophilic and small hydrophilic substances occurs mainly by diffusion, while the slower net shift of fluid between the intra and extravascular spaces takes place by convection (*cf* Landis and Pappenheimer 1963). However convection may well be of dominant importance also for the blood to tissue transport of macromole...

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., the plasma proteins, because recent data suggest that both diffusion and micro-cytosis may contribute very little to this particular transfer (e.g. Larsen, Parving and ...ing 1974, Rippe, Kamiya and Folkow 1977 Larsen 1978, Rutili 1978)

...rlier studies on the interrelationships between diffusion and convection as mechanisms ...ansfer for small hydrophilic substances through the capillary membrane are, however ...ast in part conflicting. Data by Krahoffer (1946), Pappenheimer Renkin and Borrero ...1), and more recently by Fleming and Diana (1976) suggest that the effects of con-...ion on blood to tissue transfer of most such solutes is negligible, but this view has ...n questioned by e.g. Åberg (1973). Further transport in the opposite direction, relevant ...g the elimination of tissue-produced metabolites, appears at least in skeletal muscle ...e considerably augmented by convection, ascribed mainly to a paracapillary "micro-...ing" influence (Lundgren and Mellander 1967) It should here be realized that most ...ies of blood to tissue transport usually deal with the transcapillary passage only while ...urements concerning the opposite route of transfer includes the interstitial space as ...l. Particularly in skeletal muscle this implies passage along narrow intercellular slits ... over considerable distances, since capillaries are here 40-50 μm apart, even when all ...open to flow which only occurs during intense exercise and at maximal vasodilatation.

...With respect to shifts in perfused capillary surface area in connection with vasomotor ...ustments, changes in capillary filtration capacity (CFC) and diffusion capacity (PS) ...e mainly been followed in separate investigations (e.g. Renkin and Rowell 1962 a, b ...bold et al. 1963 Kjellmer 1964 Renkin, Hudlicka and Sheehan 1966) Only in few ...dies the two parameters have been simultaneously measured with entirely independent ...hniques, as in the small intestine (Dresel, Folkow and Wallentin 1966). In the classic ...illary studies by Pappenheimer and coworkers (cf Landis and Pappenheimer 1963) ...fusion and filtration capacities were followed in close parallel by means of the isogravi-...tric technique modified to allow concomitant measurements of osmotic transients, later ...lized also by Diana, Long and Yao (1972) and Diana and Laughlin (1974). This ingenious ...osmotic transient method is, however now known to have certain limitations inherent in ...the fact that osmotic transfer of water occurs also across the endothelial cell membranes, ...ich was here balanced off by a filtration that is restricted largely to the interendothelial ...ea Fortunately this error was at least partly offset by the fact that no account was taken ...the reflection coefficient of the test solutes, which happens to introduce an error acting ...the opposite direction (cf Crone and Lassen 1970, Perl 1971)

Concerning alterations of capillary permeability only few simultaneous measurements of ...FC and PS have been performed, where Diana, Long and Yao (1972) used histamine but ...served only small histamine effects on either PS or CFC. More recently Rippe and Grega ...1978), using the presently described in vitro perfusion technique, observed marked increases ...f CFC after histamine but only minor increases in PS, results that will be further dealt with ...elow

The present study was performed in order to follow simultaneously and quantitatively ...he blood to tissue transfer of Cr-EDTA (Mw 341) and the capillary filtration capacity ...(CFC), using a colorimetric on line ...ariant of the single injection double indicator diffu-...sion method (Rippe and Stage 1976, 1978) together with a ...ariant of the isogravimetric

method (Pappenheimer and Soto Rivera 1948) The study was performed on the maximally vasodilated, artificially perfused rat hindquarter preparation in which pressures, flow and other hemodynamic parameters could be precisely controlled (Folkow *et al.* 1971). Setting out from such well controlled experimental conditions a series of graded changes in flow pressure and capillary characteristics were induced, exploring the quantitative and simultaneous effects on PS and CFC First PS for Cr EDTA was followed over a wide range of flows during maximal vasodilatation when capillary surface area and permeability were kept largely constant because the frequently observed increase in "apparent" PS with increasing flow is still a controversial phenomenon Measurements were then made at constant flow but during three principally different and selective changes in capillary [illegible] tion 1) shifts in filtration-absorption 2) alterations of perfused capillary wall area caused by graded rarifications of the capillary network, and 3) alterations of capillary permeability caused by histamine infusion In this way models of the physiologically more important variables in capillary exchange function could be separately explored as to their effects on diffusion and filtration events, under circumstances where most other parameters could be held largely constant Part of the results have been briefly reported earlier (Rippe Kamiya and Folkow 1978)

Methods

Preparation and technical arrangement Experiments were performed on the isolated hindquarters of 25 male Wistar albino rats, weighing between 270 and 380 g. The hindquarters were arranged for the "isogravimetric method" (*cf* Pappenheimer and Soto Rivera 1948 Eliassen *et al.* 1974) and for a [illegible] metric on line modification of the single injection indicator diffusion method (Rippe and Stage 1978)

The isogravimetric rat hindquarter preparation has been described elsewhere (Folkow *et al.* 1971). Briefly the tail artery was cannulated during ether anesthesia for continuous mean arterial pressure (P_A) recordings, after which the animal was further anesthetized with Nembutal[R] 3 mg/100 g, and [illegible] The abdominal aorta and the inferior caval vein were freed from the level of the renal vessels to the [illegible] lumbar ones. Then the hindquarters were isolated by mass ligatures just proximal to these latter vessels and the spinal channel and bone marrow were plugged with cotton wool soaked in silicone. Tail and paws were excluded by tight ligatures so that the preparation consisted mainly of skeletal muscle (70-75 %).

After heparinization the abdominal aorta was cannulated and connected to an artificial perfusion system driven by a Harvard perfusion pump. The caval vein was connected to a venous outflow cannula while one of the renal veins was used for continuous venous pressure recordings (P_V). The complete isolated hindquarter preparation was then placed on a balance plate connected to a strain gauge for continuous weight recordings. Finally the venous outflow cannula was coupled in series with a pH meter and a densitometer system for simultaneous recordings of Cardio-Green coupled to albumin and of Cr EDTA. The arterial inflow cannula was side branched to a step dispenser syringe for injection of the mentioned two indicators used for measurements of capillary diffusion events (*cf* Rippe and Stage 1978). The free end of the venous outflow cannula, *i.e.* the one distal to the densitometer system, could be adjusted to any desired level to set the venous outflow pressure P_V. Flow and P_A could be controlled by adjusting the setting of the perfusion pump. Tissue weight P_A and P_V were continuously monitored on a Grass polygraph, while pH and the dye dilution curves were recorded on a potentiometer writer.

4 per cent dextran (Macrodex[R] Mw about 70 000 supplied by AB Pharmacia Sweden) in Tyrode solution used as perfusate to which was added horse serum (Normal Serum, SBL, Sweden) 100 ml/L. The perfusate was gassed with a mixture of 97 % O_2 and 3 % CO_2 and kept at 38°C. Perfusion flow was kept constant at 11 14 ml/min 100 g of tissue throughout the experiment except when the effect of flow on PS were studied. Maximal vasodilatation was maintained during the whole experiment by means of slug injections (0.5 [illegible] at a time) or a slow infusion of papaverine (20 µg/ml perfusate).

Capillary filtration and diffusion were followed by frequently repeated measurements, sometimes [illegible]

ments. Small molecular permeability was assessed by measuring PS for Cr-EDTA by the calorimetric modification of the single injection indicator diffusion method (Rippe and Stage 1978). Capillary filtration conductivity was determined as capillary filtration coefficient (CFC, *cf.* Elmsen *et al.* 1974). Alterations in large molecular permeability influencing the effective colloid osmotic pressure difference across the capillary walls, were reflected by the consequent changes in capillary fluid equilibrium as they affected the net weight, even though the hydrostatic capillary pressure was kept constant.

Experimental procedures. Filtration/absorption was induced either by graded alterations of capillary hydrostatic pressure by means of changing venous pressure, or by graded changes of the crystalloid osmotic pressure by means of infusion of hypertonic saline, transiently increasing the crystalloid osmolarity from around 290 mosm/l to around 330 mosm/l at maximum. Osmotic transients of up to 30–40 mmHg were thereby created, implying a reflection coefficient for saline in the order of 0.02–0.05. More systematic measurements in this respect are, however, planned in a subsequent study.

To measure the effects on PS of changes in capillary hydrostatic pressure the following procedures were used. After 2 to 3 control measurements of CFC and PS for Cr-EDTA during complete isogravimetry venous pressure was elevated up to 30 mmHg at maximum, and after about 1 min of steady filtration PS for Cr-EDTA was measured once or twice. Flow was kept constant throughout these manoeuvres which implied a rise also in arterial pressure. After 5–7 min of filtration, venous pressure was lowered to the control value and now absorption ensued at a slightly lower rate than the former filtration. During this absorption phase, PS for Cr-EDTA was again measured and this parameter was now followed until isogravimetry was reestablished. Then additional control measurements were performed.

In applying changes in the capillary crystalloid osmotic pressure the procedures were as follows. After control measurements of CFC and PS for Cr-EDTA during isogravimetry infusion of hypertonic saline (0.3 ml/min of 4 % solution) started, upon which the preparation began to absorb fluid across the capillary walls until a new isogravimetric equilibrium was established. When the hypertonic infusion was stopped, filtration ensued until the former isogravimetric fluid equilibrium was reestablished. PS for Cr-EDTA was followed during the whole procedure, ... during the absorption phase, isogravimetric phase and the subsequent filtration phase. In some experiments also CFC was measured during the saline infusion period to check that the hyperosmolarity did not alter the capillary hydraulic conductivity.

In order to reduce the *perfused capillary surface area* in the maximally vasodilated preparation, different amounts of microspheres (ϕ 15 μm) were given as bolus injections intraarterially. In every experiment 3 to these such injections were given so as to induce graded alterations in the perfused capillary surface area. Immediately after the injections there occurred a fairly abrupt increase in arterial pressure while venous pressure remained constant, and as a consequence of the arterial pressure increase and the constant flow conditions the hydrostatic pressure rose in the perfused capillaries. Therefore, slow filtration started after a short latency (*cf.* Kamiya, Rippe and Folkow 1978). PS for Cr-EDTA, CFC and PRU_{tot} were measured at every level of plugging. By lowering venous pressure, filtration could be avoided during the measurements.

In order to induce changes in capillary permeability histamine was infused intra-arterially to the initially isogravimetric preparation in supramaximal doses (20–40 μg/ml perfusate). PS for Cr-EDTA and CFC were measured 2–3 times after about 5 minutes of histamine infusion, which caused a steady weight increase.

Calculations. The principles for the computer treatment of the on-line recorded tracer curves have been given earlier (Rippe and Stage 1978). Instantaneous extraction values (E) and integrated values (E, *cf.* Crone and Lassen 1970), plotted against time, were obtained for every set of single injection curves. E_{max} integrated up to the time when the tracer curve had declined to 50 % of peak values was used for calculations. The PS product was calculated according to the formula PS = −Qp ln (1 − E) (Renkin 1959, Crone 1963) where Qp is the measured plasma flow. This formula is valid only if capillary plasma inflow and outflow are equal, which is, of course, not the case during capillary net filtration and absorption. In these cases a simple modification of the Renkin–Crone formula was employed, based on the assumption that the capillary outflow concentration of the non permeant tracer only or alternatively that of both the non-permeant and permeant tracer is changed by the factor Q/Q′ during convection, where Q′ is the capillary outflow and Q is the capillary inflow of perfusate. The following expressions were then obtained:

$$PS = \frac{Q \cdot E \cdot \ln \frac{Q}{Q'}(1 - E)}{1 - \frac{Q}{Q'}(1 - E)} \approx -\frac{Q + Q'}{2} \ln (1 - E)$$

The expressions reduce to the simple Renkin–Crone formula if Q is set equal to Q The difference between the exact formulation (left) and the approximate one (right) is at maximum 0.5 for the filtration rates and extraction values of this study which was evident from a simulation procedure [illegible] Q [illegible] and E were changed over a wide range

As also the non-permeant tracer is slightly permeable through the capillary all, corrections [illegible] made for this. The filtration-dependent transcapillary plasma clearance of albumin (Cl_{alb}, [illegible]) during dextran—horse-serum perfusion at maximal vasodilatation has earlier been determined [illegible]

$$Cl_{alb} = 0.05 + 0.43\,Jv$$

where J is the net filtration rat (Rippe and Folkow 1977). As this relation was known, the [illegible] albumin could easily be determined at every filtration rate at known perfusate flow (Qp) from the [illegible] $Cl_{alb} = Q_p\ E_{alb}$. The measured extraction values for Cr EDTA at peak time E_{peak} (see Fig. 2) [illegible] corrected according to the formula

$$E_{corr} = \frac{E_{alb} + E_{peak}}{1 + E_{alb}}$$

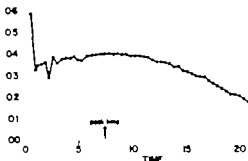
The ratio E_{corr}/E_{peak} was used as a correction factor for E_{area}. Corrected E_{area} was then [illegible] the modified Renkin–Crone formula above. Though negligible at low filtration rates, this correction [illegible] comes increasingly important at high filtration rates.

Correction for histamine induced transcapillary leakage of the non-permeant tracer was not [illegible] this study since no experime tal data were available for such a correction. However the error [illegible] making this correction could be roughly estimated from the data on albumin clearance during [illegible] infusion by Appelgren, Jacobsson and Kjellmer (1966) and on control albumin clearance at [illegible] during dextran-horse serum perfusion (see above) and pure horse serum perfusion (cf Rippe and Folkow 1977 Rippe Kamiya and Folkow to be published). The results of the present study [illegible] accou t. Albumin clearance by large pore diffusion was assumed to increase from around [illegible] ml/min 100 g (Rippe Kamiya and Folkow to be published) to about 0.3 ml/min 100 g by [illegible] and the weighted reflection coefficient for th whole capillary membrane to decrease from around [illegible] around 0.15–0.20 (see Results). Dextran-horse serum perfusion alone induces a decrease in the [illegible] reflection coefficient f r albumin from around 0 90–0 95 during perfusion with pure horse serum [illegible] Pappenheimer and Soto R vera 1948) t around 0.5 (Areek al 1969 Rippe and Folkow 1977) [illegible] by affecting the small pores without much changing their dimensions (cf Rippe and Folkow 1977). [illegible] on such considerations albumin clearance during histamine infusion was estimated to. Cl_{alb} [illegible] where the symbols were defined above The error of not correcting the E alues according to this [illegible] results in an underestimation only of some 7 15 in the present study Most PS measurements [illegible] histamine infusion were perf rmed at lowest possible f ltration rates (around 0 5 ml/min × 100 g) which [illegible] brought bout by e g lowering venous pressure

Results

1 *Apparent fractional extraction as a function of time*

In most experiments the apparent fractional extraction Cr EDTA *versus* time showed [illegible] rapid initial rise, directly followed by a transient, dip-like decrease, but at the time when the tracer curves reached their peaks ('peak time) a plateau of some seconds duration was obtained, followed by a more gradual decrease (Fig. 1) In some 40 % of the experiments the initial apparent fractional extraction value was slightly higher than that at peak time (E_{peak}), in 30–40% it was approximately the same and in around 30% it was somewhat lower Maximum value of the overall extraction, calculated by the area method (E_{area}) used in this study (cf Rippe and Stage 1978) was some 5–8% lower than the maximal instantaneous extraction (E_{max}) but roughly proportional to it (Fig. 2). Thus in this study

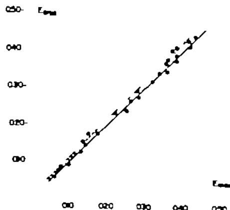


Computer plot of apparent fractional ... of Cr-EDTA vs. time from ... representative experiment.

...ative changes in area extraction, or in PS calculated from E_{area} paralleled the changes ... which, on the basis of computer modelling, has been proposed to be the best ap...imation of true overall extraction (*cf.* Bassingthwaighte 1974). However E_{area} was in ...resent study preferred to E_{max} because of its higher reproducibility in the given experi...ntal situation. Thus the coefficient of variation between sequential E_{area} determinations ... only between 2 and 3 %, while that of E_{max} was around 5 %.

Apparent PS as function of perfusate flow

...hough the vasculature was maximally dilated and thus all capillaries were open to flow ...also judged from CFC determinations, apparent PS varied with perfusate flow in the ...er range as shown in Fig. 3. However, at flows above 11–12 ml/min 100 g PS remained ...ntially constant, even though a slight tendency of increase was seen also here. For such ...sons the PS determinations, performed after induced changes in capillary function, were ... performed at flows above 11 ml/min 100 g and flow was in the individual experiment ...t constant throughout so that the PS determinations should not be disturbed by flow ...nges. At a mean flow of 12.5 ± 0.3 ml/min 100 g and a mean E_{area} of 0.365 ± 0.001



...ig. 2. Relationship between maximal instan...neous extraction (E_{max}) and area extraction ...(E_{area}), integrated up to the time ... when the tracer ...urves had declined to around 50 ... of their peak ...alues in 9 randomly selected expts. The data are ...aken from control recordings during maximal ...modilatation, as well as from recordings during ...he different test manoeuvres of this study. The ...ntity line (hatched) is shown together with the ...ne of the linear regression of E_{area} on E_{max} ...escribed by the equation E_{area} ... E_{max} ... 0.973 ...0.007

The expressions reduce to the simple Renkin–Crone formula if Q is set equal to Q′. The difference between the exact formulation (left) and the approximate one (right) is at maximum 0.5 ... for the filtration rates and extraction values of this study which was evident from a simulation procedure when Q ... and E were changed over a wide range.

As also the non-permeant tracer is slightly permeable through the capillary wall, correction had to be made for this. The filtration-dependent transcapillary plasma clearance of albumin (Cl_{alb}, ml min ...) during dextran—horse-serum perfusion at maximal vasodilatation has earlier been determined as

$$Cl_{alb} = 0.05 + 0.43\ J$$

where Jv is the net filtration rate (Rippe and Folkow 1977). As this relation was known, the extraction of albumin could easily be determined at every filtration rate at known perfusate flow (Qp) from the relation $Cl_{alb} = Q \cdot E_{alb}$. The measured extraction values for Cr EDTA at peak time E_{peak} (see Fig. ?) were corrected according to the formula

$$E_{corr} = \frac{E_{alb} + E_{peak}}{1 + E_{alb}}$$

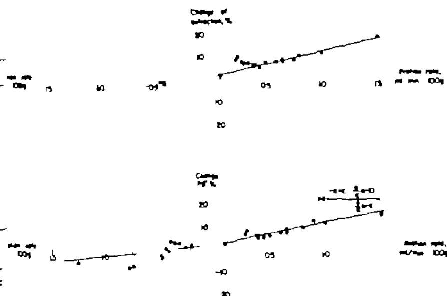
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Results

1. *Apparent fractional extraction as a function of time*

In most experiments the apparent fractional extraction Cr EDTA versus time showed a rapid initial rise, directly followed by a transient, dip-like decrease, but at the time when the tracer curves reached their peaks ("peak time") a plateau of some seconds duration was obtained, followed by a more gradual decrease (Fig. 1). In some 40 % of the experiments the initial apparent fractional extraction value was slightly higher than that at peak time (E_{peak}), in 30–40 % it was approximately the same and in around 30 % it was somewhat lower. Maximum value of the overall extraction, calculated by the area method (E_{area}) used in this study (cf. Rippe and Stage 1978) was some 5–8 % lower than the maximal instantaneous extraction (E_{max}) but roughly proportional to it (Fig. 2). Thus in this study



4 The relative changes in extraction (E) and apparent PS for Cr-EDTA at graded alterations in capillary filtration-absorption. Filled circles are from experiments in which convection was induced by changing capillary hydrostatic pressure, and open circles from experiments in which such alterations were induced by changing intracapillary osmotic pressure. The hatched line (upper part) shows the change in extraction which is caused by convection-induced differences in (arterial) inflow to and (venous) outflow from the exchange vessels. The changes in transport-constant for C -EDTA are of the order of magnitude as predicted from the theory of irreversible thermodynamics

...re Js is the measured unidirectional flux of solute, J is the filtration rate, $\bar{c}$ the mean ...capillary solute concentration and the reflection coefficient of the solute. Interstitial ...centration is here supposed to be zero. The first term of this expression gives the pure ...usion flux and the second one the filtration flux. The obtained data are well compatible ...h this expression for a σ of Cr EDTA in the order of 0 05–0.15 and for a mean solute ...centration in the pore that is higher than $\bar{c}/2$ during filtration and lower than this value ...ring absorption (*cf* Grotte 1956). No attempt was made to calculate σ because the ...tistical errors inherent in the data will then be amplified in an unacceptable manner

The extraction values during filtration-absorption are affected both by pure convection ...ects on total transport (see above) and by convection induced concentration/dilution ...fects, causing artefactuous changes of the venous concentrations of permeant and non-...fusing tracer In Fig. 4 this artefactuous *relative* change in E is represented by a hatched ...line, which was calculated according to the formula

$$\Delta E \quad \left\{1-\exp\left[PS\Big/\left(Q-\frac{Jv}{2}\right)\right]\right\}\Big/\{1-\exp-(PS/Q)\}$$

...here Jv is the volume flux through the capillary membrane and (Q − Jv/2) represents the ...ean perfusate flow through the system (see Methods). The numerator represents the E ...alue corrected according to the modified Renkin–Crone formula used in this study (see

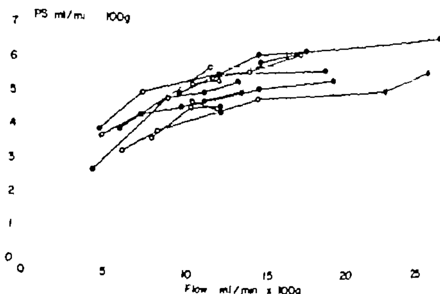


Fig. 3 Apparent PS for Cr EDTA in the maximally vasodilated hindquarter vascular bed plotted against flow in 13 e pts. Values from the same expt. are interconnected by straight lines. PS shows a slight tendency of flow dependence at flows below some 11 12 ml/min 100 g. In contrast, CFC is largely independent of flow in this state of maximal vasodilatation.

mean PS during control was in 25 experiments 5.67 ± 0.13 ml/min 100 g. In every experiment at least 6 control determinations of PS were performed.

3 *Apparent PS versus rate of filtration-absorption*

Whenever net filtration or absorption occurs, transcapillary solute transfer will to some extent be modified as shown in this study. The solute transfer could therefore be regarded as composed of a "pure" diffusion term (PS) and a convection term and they may together be called "apparent PS (PS')". Concerning the present interaction between diffusion and filtration-absorption it should be stressed that only the *blood to tissue* diffusion transfer was dealt with because of the technique employed for diffusion studies. As briefly discussed in the Introduction this is not necessarily equivalent with the reversed transfer of cell produced metabolites or of a tracer that is uniformly dispersed in the interstitial space, which in *e.g.* skeletal muscle is narrow and with considerable intercapillary distances. The changes in extraction and in apparent PS were plotted against the rate of filtration-absorption as shown in Fig. 4. PS calculated from the modified Renkin–Crone formula, derived from the condition when perfusate inflow and outflow differed (see Methods), showed a slight but significant *increase* with increasing filtration and also a tiny decrease with increasing absorption. The slope of the linear regression of percentual increase in apparent PS on filtration rate was 9.47 ± 2.18 % ml min 100 g ($r = 0.66$) and the slope of the percentual decrease in apparent PS on absorption rate was 4.28 ± 1.81 % ml min 100 g ($r = 0.48$). The ordinate intercept was 1.78 ± 1.30 % and 2.31 ± 1.46 %, respectively.

In accordance with the formulation by Kedem and Katchalsky (1958) the transfer of solute during filtration might be expressed as

$$PS\ \bar{c} - Js = PS\bar{c} + Jv\frac{\bar{c}}{2}(1 \quad \delta)$$

5. The relative changes in PS on histamine infusion vs. the simultaneously recorded relative changes C. The dotted lines show the theoretical relationship between the parameters at A) an induced increase mber but not in radius of the small pores (r ≈ 40–60 Å), B) an increase in radius but not in number of l pores, C) an increase in number of the relatively few large pores (r ≈ 250 Å, Grotte 1956), D) an se in radius but not in number of large pores, assuming large to small pore number ratio of 1 30 000 te 1956).

ted by papaverine, the PS changes could be quite marked upon even moderate smooth cle adjustments and consequent changes of flow resistance.

n contrast to the considerable changes in PS on even modest changes in the resistance to as result of microvascular plugging, CFC always changed less. The observation that CFC changes are usually less extensive than the resistance changes on e.g. metabolic odilatation is evident also from earlier studies (e.g. Cobbold *et al.* 1963 Kjellmer 1964), ere the present relationship between CFC and resistance closely resembles that presented Kjellmer 1964 in connection with increasing metabolic vasodilatation in cat muscle.

c Capillary diffusion and filtration capacity at histamine-induced permeability changes

e initially isogravimetric hindquarter preparation responded to histamine infusion (5–40 /ml of perfusate) with often marked increases in CFC and in net transcapillary fluid tration, causing weight increases of the preparation. The maximal CFC values recorded ere some 0.20 ml/min mmHg 100 g, compared with around 0.04 ml/min mmHg 100 at complete vasodilatation without histamine, and the maximal filtration rates were round 1.8 ml/min mmHg 100 g. These maximal permeability increases to histamine ccurred at doses around 20 µg/ml perfusate. Depending on the quality of the horse serum, sed together with the dextran as perfusate colloids, there was relatively great variations in

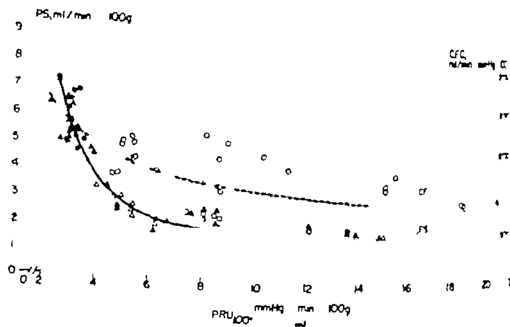


Fig. 5 CFC and PS-Cr-EDTA versus peripheral resistance at different levels of capillary plugging (microspheres $\phi \approx 15$ μm) during constant flow and maximal dilatation. Control values are CFC = ... 0.001 (SE) (ml/min mmHg × 100 g) and PS = 5.67 ± 0.13 (ml/min × 100 g). The relationships would follow the dotted line if the decrease in number of perfused capillaries were proportional to the increase in peripheral resistance during plugging. — PS seemed to change as theoretically predicted, whereas CFC changed less. Filled symbols control values. Open symbols test values.

Methods) and the denominator the uncorrected E value according to the original Renkin-Crone formula (e.g. Renkin 1959). Uncorrected E value was 0.365 as PS was set at 5.6 and Q at 12.5. For the matter of simplicity the solvent drag component of PS was set at zero, i.e. PS was held constant. Even if PS is allowed to change during convection, the relative change in E by concentration/dilution effects by convection is with a very good approximation, the same as that shown in Fig. 4.

4 *Relationships between capillary diffusion and filtration capacities at alterations in capillary surface area*

In Fig. 5 PS and CFC values are plotted against resistance to flow at maximal vasodilatation and at constant flow (around 12–13 ml/min 100 g) first, during the control situation and second, at different levels of rarification of the capillary bed by means of i.a. injected microspheres. The dotted line represents the expected decrease in both parameters in case the reduction in capillary surface area were exactly proportional to the increase in peripheral resistance. This would be the case only if vascular dimensions were not affected by the pressure increase secondary to microvascular plugging at constant flow and if this plugging excluded identical proportions of resistance and exchange vessels, i.e. if there were no more precapillary interconnections below the level of plugging to allow for flow redistribution within the exchange network. On the whole, the PS changes seemed to follow the theoretical line relatively closely. Likewise, during control and before the vessels were fully

lar beds, which is practically impossible when complete uniformity is not at hand, but to study the *relative* changes in 'apparent' PS in different standardized situations, ting in a model fashion principally important changes in microvascular function. To e flow heterogeneity to a minimum during control, the capillary bed was maximally d with papaverine, stagnant flow was avoided by using cell free perfusate and flow cept reasonably high, *i.e.* between 11 and 14 ml/min 100 g which is 3 times higher in resting steady state for muscle.

parent PS for Cr-EDTA increased with increasing flows and this tendency was most ent at flow below some 10 ml/min 100 g. This phenomenon, frequently observed (*e.g.* Renkin 1959 Yipintsoi *et al.* 1970, Trap-Jensen and Lassen 1970 Linde, Chis- and Rosell 1974, Paaske 1977), here occurred even though the vascular bed was maxi- dilated as checked by measurements of CFC. Thus, the flow-dependent increase in rent PS in this study *cannot* be explained by any increase in perfused capillary surface. In other studies where capillary surface area was not controlled, recruitment of capil- may of course, partly explain the PS dependence of flow (*cf.* Paaske 1977). Other anisms behind this phenomenon is probably increasing heterogeneity of microvascular hen perfusion pressure and flow decreases to low levels, resulting in semistagnant itions in some *per se* open loops which therefore hardly at all contribute to tracer ance. Further the chances for an increasing contribution of early back diffusion to the minations of the extraction fraction may for the same reasons increase at low flows. ever the impact of such factors on the PS determinations were here minimized when effects of changes in surface area and permeability were tested, because flow was then ys kept well above the level of substantial flow limitation for diffusion, as mentioned er

he question whether convection is important or not as a mechanism for the blood to e transfer of small hydrophilic molecules has been matter of some controversy over last few decades. Transcapillary convection implies a direct transport of solute ac mpanying the flow of solvent through the capillary pores, *i.e.* solvent drag. Also, convec- might indirectly increase the flux of solute through the membrane by *e.g.* improving ring of the interstitial fluid, and thus preventing interstitial gradients to build up. By mechanism the interstitial concentration would be lowered and diffusion across the illary wall augmented according to Fick's law In addition, the transmembrane diffusion dients might be changed also by concentration/dilution effects which are produced when ute is reflected to a greater extent by the microvascular membrane than water during ration/absorption.

With the single injection technique it is possible to study the solvent drag effects and the the concentration/dilution effects on solute transfer In this study correction was made the impact of the last-mentioned factor by means of estimating solute transport from a odified Renkin–Crone formula, which takes into consideration convection-induced nges in flow through the system (see Methods). However in this blood to tissue transfer erstitial stirring effects would not cause changes in extraction, at least not under ideal nditions, because the extraction determination is made from the first few seconds of the ngle injection curves (around peak time) when tissue concentration is supposed to be close

the extent of maximal histamine response among individual experiments. Quite unexpectedly the greatest shifts in permeability by histamin were obtained if "aged" horse serum (stored for 3 weeks at +4 C) was used in the perfusate This is probably due to the fact that fresh horse serum contains functional histamine antagonists with respect to capillary permeability, such as catecholamines as dealt with in another study (Rippe and Grega 1978).

In contrast to the gross changes in capillary fluid equilibrium and CFC induced by histamine, PS for Cr EDTA changed very little. The range of PS increment was of the order of 0–20% from control as shown in Fig. 6 It is, however important to realize that solute transport in these experiments is somewhat underestimated because of histamine induced leakage of the nonpermeant tracer The error of not taking this factor into account results in an underestimation of solute transfer of 7–15 %, as discussed under Methods. Therefore, the maximal change in *total* transport of Cr EDTA after histamine is some 20–30%, of which about two thirds is the result of increased diffusion This is to be compared with the observed increase in CFC of maximally 400–500 %.

Discussion

The single injection indicator diffusion method has been used extensively to assess the unidirectional blood to tissue transfer of various molecules (*e g* Crone and Lassen 1970; Bassingthwaighte 1974), but only seldom has it been possible to combine it with other methods for functional capillary studies Therefore the present study was performed, combining this method with the isogravimetric technique (Pappenheimer and Soto Rivera 1948) to allow simultaneous measurements of diffusion and filtration in the rat hindquarter vascular bed during controlled artificial perfusion when pressure, flow pre- and postcapillary resistances, capillary surface area, capillary permeability *etc* could be more or less independently changed to a known extent

From most earlier single injection studies it is evident that the curve representing the fractional extraction *versus* time (E(t)) is highly variable in shape depending on the tracer studied, the sampling system employed, flow rate, etc. In the present study the most common shape of the E(t) curve was a rapid initial increase followed by a diplike decrease and then, after tracer curve peak time, by a plateau lasting for some seconds to again decrease gradually. The most likely explanation for the initial peak in E(t) is Taylor diffusion (*cf* Taylor 1953), while the most probable mechanism behind the second slower rise in E(t) preceding the plateau is heterogeneity in capillary arrangement and hence in flow Heterogeneity in this sense is however a normal feature of all capillary networks, even in the relatively uniform myocardial vascular bed where rising E(t) curves have also been registered (*e g* Duran and Kirk 1970 Bassingthwaighte Yipintsoi and Grabowski 1975) The presence of such heterogeneity inherent in the very arrangement of capillary networks and supply routes implies that when out-diffusion starts in some capillaries, back diffusion may have already started in others. These normal consequences of how vascular beds are designed tend, however, to reduce the experimentally recorded extraction fraction and will thus underestimate the real permeability surface area (PS) product The aim of the present study was, however, not to give an absolutely precise estimation of the 'true PS product for Cr EDTA in mod

r beds, which is practically impossible when complete uniformity is not at hand, but to study the *relative* changes in 'apparent' PS in different standardized situations, ing in a model fashion principally important changes in microvascular function. To flow heterogeneity to a minimum during control, the capillary bed was maximally l with papaverine, stagnant flow was avoided by using cell free perfusate and flow pt reasonably high, *i* between 11 and 14 ml/min 100 g which is 2–3 times higher o resting steady state for muscle.

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to zero Therefore, to study interstitial stirring effects it is far more rational to follow the diffusion from the interstitium to blood with other techniques (cf Lundgren and Mellander 1967) or by using the 'tail' of the single injection curves when the indicator returns back from the tissue spaces to the blood stream (Paaske 1977 Kamiya, Rippe and Folkow unpublished results) Another possibility to study stirring effects would be to use the constant infusion technique by which it might be possible to assess both capillary membrane, interstitial diffusion and cell membrane impedances (Sheehan and Renkin 197 The conclusion that might be drawn from the present data is that convection modifies the blood to tissue transfer of solute to an extent that can be theoretically predicted from the theory of irreversible thermodynamics (Kedem and Katchalsky 1958). This is in agreement with the results from recent single injection measurements on the blood perfused, maximally vasodilated dog hindlimb (Fleming and Diana 1976) With the present data it is, however, not possible to give an accurate estimation of the reflection coefficient of Cr-EDTA

In essence, the present results imply that convection is normally *not* a quantitatively important mechanism for the transport of small molecular weight solutes across the capillary membrane *per se* Even at heavy muscular exercise, when the filtration rate might amount to 0 5–1 ml/min 100 g of tissue, the contribution of filtration to the transcapillary transport of small molecules from blood to tissue is below 10 of the total transport obtained for Cr EDTA (Mw 341) This convection contribution to transport is theoretically still less for molecules smaller than Cr EDTA

Upon reducing the capillary surface area by microvascular plugging PS for Cr-EDTA as measured during constant flow and maximal vasodilatation, was markedly reduced but the reductions of CFC were always relatively smaller Both relatively and absolutely the PS reduction was most pronounced at changes in resistance to flow from 2.8 to around 8 PRU (mmHg ml min 100 g), these resistance figures being valid for a perfusate viscosity slightly above two thirds of that for blood *in vivo* Fig. 4 shows the asymptote describing the theoretical relation between PS and resistance to flow if vascular dimensions were not affected by the transmural pressure increase produced by microvascular plugging and if there were no collateral interconnections between this level and the exchange vascular section The equation of this theoretical function is (PS/5 67) = (2.8/R) where R is the present resistance per 100 g of tissue (PRU) and 2.8 is the PRU at maximal vasodilatation where PS is 5 67 ml/min 100 g. The vascular distension occurring when resistance and pressure is increased as a result of plugging, tends to lower the PS *vs* R relation below the theoretical line Further there are probably some collateral interconnections between the plugging sites and the exchange vessels by which relatively more capillaries can be reached particularly at higher pressures, giving higher PS values at high PRU values than predicted by the theoretical curve In addition, solute transfer studied with the dual indicator single injection method might also be affected by the fact that heterogeneity in microvascular perfusion increases with plugging, whereas the chances of early back diffusion decrease

When comparing the present PS data with those for Cr EDTA (Mw 341) or sucrose (Mw 342) obtained in other laboratories the hemodynamic state of the preparation as well as species variations must be taken into account At maximal vasodilatation PS for Cr-EDTA would be some 5 5–6 ml/min 100 g in rat cat and dog muscle vasculature (Paaske 197

-ing and Dama 1976) and in the resting state around 1 1.5 ml/min 100 g (recalculated : Crone 1963 on cat, assuming a resting plasma flow of 4 ml/min 100 g), while at sub-
imal flows during work hyperemia the values would fall inbetween (Trap-Jensen and -en 1970, man s forearm). Assuming a capillary surface area of 7 000 cm² (*cf* Landis -Pappenheimer 1963) at maximal vasodilatation a permeability value (P_d) for sucrose or
EDTA of some 1 4–1.5 10^{-4} cm/s is obtained.

Concerning diffusion exchange the relative shift of perfused capillary surface area in etal muscle from rest t maximal vasodilatation during heavy exercise would thus be o about 5 times (Fig. 5). However when estimated from CFC determinations this figure nly around 3 times, according to Fig. 5 The capillary surface area available for *diffusion* -ange in the resting state thus seems t be overestimated when judged by CFC measure-
its. The main reason seems to be that during a CFC determination the capillary pressure ation transmits also to capillaries where flow is too low to be of significant relevance for d *diffusion* exchange, but where the far less flow-limited filtration process still can take e (*cf* Folkow and Mellander 1970). For such reasons, PS estimations provide the most vant picture of the number of capillaries available for effective *diffusion* exchange of rients, hile CFC measurements as precisely reflect the capillary surface available for *fluid* exchange between intra- and extravascular spaces, which for .g plasma volume dation is the relevant thing. These two types of measurements thus reflect two biologically portant but different aspects of the transcapillary exchange processes. When these pro-
ses ere earlier measured separately marked changes in diffusion capacity as a response reduction in capillary surface area have usually been bserved (.g Renkin and Rosell 52 a). However the changes in CFC on *e.g* metabolic vasodilatation in skeletal muscle ve usually been observed to be relatively smaller (*cf* Cobbold *et al.* 1963 Kjellmer 1964), ich is in agreement with the present measurements of both processes simultaneously

Histamine infused into the hindquarter vascular bed markedly increased CFC and pro-
ced net fluid filtration in the initially isogravimetric preparation, but did not alter vascular istance in the already maximally asodilated preparation. This marked fluid f ltration ust therefore be due to a decrease in effective transcapillary colloid osmotic pressure differ ce or in other words, to marked decrease of the reflection coefficient for the perfusate acromolecules (dextran, 70 000, and albumin). In this situation histamine produced ximal filtration rate of 1 7 1.8 ml/min 100 g and CFC increased from around 0.04 a maximum 0.2 ml/min mmHg 100 g Dividing 1 7 by 0 2 gives the maximal decrease effective colloid osmotic pressure produced by histamine in these experiments, .e 8 5 mmHg.

The present relative changes in both CFC and isogravimetric capillary pressure (P_{ci}) re lower than those reported by McNamee and Grodins (1975) probably because the esent measurements set out from the maximally asodilated state where the greater number of perfused capillaries implies that CFC is around three times higher than in the resting steady state It cannot be ascribed to any initially bnormal permeability increase to water or small molecules in the present preparation, as judged from the CFC and PS data. However the permeability to macromolecules is somewhat increased during dextran perfu-
sion by unknown mechanisms (Areekul 1969 Rippe and Folkow 1977), and this may be the

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$$PS_{test}/PS_{control} - 100 \quad \left(\frac{r}{r_L}\right) \quad (CFC_{test}/CFC_{control} - 100)$$

re all parameters except (r_s/r_L), which is the ratio of radii of small to large pores, are ained in the Figure. (See appendix for derivation.) From this equation it is evident that r −40 and 250 (Grotte 1956), a fourfold CFC increase which would occur if the ber of large pores increased 60 times, assuming one large pore in 30 000 small pores e control situation, would increase PS only 7 7%. Both large pore and small pore radii today often considered to be somewhat larger (e.g. Wiune 1965 Paaske 1977) than the es used in the calculation above. Therefore, the r_s/r value used in Figure 6 may be too and a lower value will shift the relation towards the abscissa.

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$$PS_{test}/PS_{control} - 100 \quad \sqrt{100(CFC_{test}/CFC_{control} - 100)\frac{n_l}{n_s}}$$

re n_l/n_s is the ratio of number of large to small pores. It describes the relation of relative and CFC changes when the radii of the large pores are uniformly increased, while the ll pore radii remain constant. At a CFC increase up t 400%, $PS_{test}/PS_{control}$ would rease only by 1%, using a value of n_l/n_s of 1/30 000 for the calculations. A still smaller n_l/n_s ratio (*cf* Wiune 1965 Renkin, Carter and Joyner 1974) would shift also this relation wards the abscissa

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To summarize, it was in the present study possible to follow diffusion and filtration events ith highly reproducible, simultaneous measurements in a well controlled, constant-flow rfused model preparation over a wide range of induced changes in capillary surface area, ermeability and fluid filtration. Hopefully the data from this study have shown that rther investigation of capillary membrane characteristics in other vascular beds and tissues y such an approach with combined techniques is needed for full understanding of the echanisms behind the transcapillary exchange of molecules.

Appendix

Derivation of the equations for lines C and D in Fig. 6

The values obtained during histamine infusion on $PS_{test}/PS_{control}$ (*) were plotted against the simultaneously obtained values on $CFC_{test}/CFC_{control}$ ( ) as show in Fig. 6. According to the 'double pore'

reason why the present P_t decrease was slightly lower than that obtained by McNamara and Grodins

P_{ct} for this dextran-horse serum—Tyrode perfused preparation has earlier been determined to 12.4 mmHg (Kamiya, Rippe and Folkow 1978) and the reflection coefficient (σ) for the perfusate colloids is then around 0.5 (Areekul 1969 Rippe and Folkow 1977). According to the modified Starling theory the following is valid

$$P_{ct} = P_i + \sigma(\pi - \pi_i)$$

where P_{ct} and σ were defined above and P_i is the interstitial fluid pressure, while π and π_i are the colloid osmotic pressures in the perfusate and the interstitium, respectively. For the sake of simplicity P_i could be set to zero (*cf* Eliassen *et al.* 1974) Knowing the P_{ct} values before and after histamine and control σ a rough estimation of σ for the perfusate colloids during histamine is obtained if P_i and π_i are assumed to remain largely constant, which would approximately hold for the first few minutes of histamine infusion. σ for the perfusate macromolecules during histamine infusion would then be around 0.15 and had thus been reduced markedly from control when it was around 0.5

In contrast to the pronounced changes in CFC and macromolecular permeability during histamine infusion, PS for Cr EDTA changed very little (*cf* Rippe and Grega 1978) This is in principal agreement with findings by *e g* Diana, Long and Yao (1972) although the changes also in CFC and P_{ct} were much smaller and more transient. In the great majority of experiments on canine adipose tissue Linde, Chisholm and Rosell (1974) noted only small changes in PS for sucrose on histamine infusion, around 25% increase. Further the failure to show substantial effects of histamine on small molecular permeability has been experienced by other investigators (Lassen, personal communication).

This discrepancy between the marked histamine-induced changes in hydraulic conductance and large molecular permeability on the one hand, and the almost insignificant increase of small molecular permeability on the other can best be explained by a preferential action of histamine on venular gap formations (large pores), increasing their number or/and their radii as illustrated in Fig. 6 This Figure also illustrates the theoretical relations between the two parameters in four different situations *A* At an increase in the number of small pores *B* At a uniform increase in small pore radius. *C* At a conversion of a minor fraction of small pores to large pores (r ~ 250 Å) or simply opening more large pores, and *D*, at an increase in radius of preexisting large pores. Vesicular transport as a possible mechanism of solute transfer was not considered here, partly because the present data cannot be explained by this mechanism for reasons mentioned below partly because more direct evidence against this mechanism for transcapillary macromolecular transfer was recently advanced (Rippe, Kamiya and Folkow 1977)

Line *A* assumes a linear increase in number of small pores with parallel increases in PS and CFC. Line *B* assumes a uniform small pore radius increase, increasing hydraulic conductivity to the fourth power of the radius (Poiseuille's law) and small molecular diffusion capacity (nonrestricted diffusion) to the second power of the radius (Fick's law). Line *C* is a variant of line *A* where the number but not the radius, of large pores increases The equation for this line is

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theory (Grotte 1956) the percentual change in CFC_{test} and PS_{test} from control (100) during histamine infusion can be expressed according to Poiseuilles and Fick's laws, respectively (assuming non-restricted filtration/diffusion) as

$$\frac{CFC_{test}}{CFC_{control}} = 100\,\frac{n_l r_l^4 + n_s r^4}{n_s r^4} = 100\left[\frac{n_l}{n_s}\left(\frac{r_l}{r}\right)^4 + 1\right] \quad (1)$$

$$\frac{PS_{test}}{PS_{control}} = 100\,\frac{n_l r_l^2 + n_s r^2}{n_s r^2} = 100\left[\frac{n_l}{n_s}\left(\frac{r_l}{r_s}\right)^2 + 1\right] \quad (2)$$

Here n_l/n_s is the ratio of number of large to small pores, r_l/r the ratio between their radii. $CFC_{control}$ and $PS_{control}$ represent the PS and CFC values in the theoretical situation where large pore filtration and diffusion are zero. In reality CFC, and to some extent PS, during control are slightly larger than the theoretical control values because of a minor contribution of large pore filtration and diffusion transport even before histamine infusion (Grotte 1956, see further below). Eq. (1) and (2) give

$$\frac{PS_{test}}{PS_{control}} - 100 = \left(\frac{r}{r_l}\right)^2\left(\frac{CFC_{test}}{CFC_{control}} - 100\right) \quad (3)$$

Eq. (3) is the equation for line C in Fig. 6. Here n_l/n_s is allowed to change while r_l/r is set constant. Changing n_l/n_s from 1/30 000 (Grotte 1956) to 59/30 000 induces a change in $CFC_{test}/CFC_{control}$ from 105 to 400 according to Eq. (1), while $PS_{test}/PS_{control}$ increases from 100.1 to 107.7, according to Eq. (2) and (3) assuming a value on r_l/r of 6.25 (Grotte 1956).

Rearranging Eq. (2) gives

$$r^2 = 100\,\frac{n_l}{n_s}\,r_l^2\left(\frac{PS_{test}}{PS_{control}} - 100\right) \quad (4)$$

Substituting Eq. (4) in Eq. (3) gives

$$\left(\frac{PS_{test}}{PS_{control}} - 100\right)^2 = 100\,\frac{n_l}{n_s}\left(\frac{CFC_{test}}{CFC_{control}} - 100\right) \quad (5)$$

The positive square root of both sides of Eq. (5) gives the equation of line D in Fig. 6. Here r_l/r is allowed to change while n_l/n_s is set constant. Changing r_l/r from 6.25 (see above) to 17.32 induces a change in $CFC_{test}/CFC_{control}$ from 105 to 400 according to Eq. (1), while $PS_{test}/PS_{control}$ changes from 100.1 to 101.0 according to Eq. (2) and Eq. (5), assuming a value on n_l/n_s of 1/30 000 (see above).

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rom the Laboratory of Clinical Biochemistry University of Bergen, Haukeland Sykehus, Norway

On the capacity of the β-oxidation of palmitate and palmitoyl-esters in rat liver mitochondria

By

MIKAEL FARSTAD and ROLF BERGE

Received 29 March 1978

Abstract

STAD, M. and R. BERGE. *On the capacity of the β-oxidation of palmitate and palmitoyl-esters in rat liver mitochondria* Acta physiol. scand. 1978 *104* 337 348

β-oxidation of palmitate, palmitoyl-CoA and palmitoyl-L-carnitine proceeded at high rate in isolated rat mitochondria. At high concentrations (100 nmol/mg protein) the oxidation of palmitate and palmitoyl-CoA was only partly carnitine dependent. All substrates were most rapidly oxidized in the presence of [illegible] and state 3 conditions. Succinate inhibited β-oxidation especially in state 4 conditions. β-[illegible] was faster in hypotonic than in isotonic medium both in state 3 and state 4 conditions. Hyper-[illegible] inhibited β-oxidation. The initial formation of palmitoyl-CoA from palmitate CoA and ATP [illegible] than the oxidation of palmitate under identical conditions The presence of bovine serum albumin [illegible] the β-oxidation, especially with palmitoyl-CoA or free palmitate as the substrates. Mitochondria [illegible] palmitoyl-CoA hydrolase which may influence the available extramitochondrial palmitoyl-CoA. [illegible] present results demonstrate no single rate limiting step in the β-oxidation in vitro Both the NADH/[illegible]D ratio, competition for the respiratory chain, the level of ADP binding of palmitoyl-CoA to extra-[illegible]chondrial protein, and possibly extramitochondrial hydrolysis of palmitoyl-CoA all seem to influence [illegible] rate of β-oxidation in vitro. It is suggested that in vivo the most important factor is the availability of [illegible]l-CoA to the outer carnitine palmitoyl-transferase of the mitochondria.

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[illegible] oxidation of fatty acids includes the formation of acyl-CoA, transfer of the acyl-group [illegible] carnitine with subsequent translocation of the acyl-carnitine through the inner mito-[illegible]ondrial membrane, acylation of intramitochondrial CoA, entrance of acyl-CoA into the [illegible]oxidation cycle and transfer of the electrons through the electron transport chain. Any of [illegible] steps could be rate-limiting, either by the activity of the involved enzymes, by the [illegible]ilability of competing substrates or by competition for cofactors such as CoA or NAD [illegible] Previously published results have indicated that the fatty acid activation (Schultze *et al.* [illegible]72, Flatmark and Pedersen 1975 Oram *et al.* 1973), the synthesis or translocation of [illegible]yl-carnitine, (Oram *et al.* 1973 Shephard *et al.* 1966, Fritz 1968 McGarry *et al.* 1973) [illegible] capacity of the β-oxidation cycle or the capacity of the electron transport chain (Pande [illegible]971) might be the rate limiting step Several of the involved reactions may vary under [illegible]fferent experimental or metabolic conditions in different organ systems from various

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The oxidation of fatty acids includes the formation of acyl-CoA, transfer of the acyl-group to carnitine with subsequent translocation of the acyl-carnitine through the inner mitochondrial membrane, acylation of intramitochondrial CoA, entrance of acyl-CoA into the β-oxidation cycle and transfer of the electrons through the electron transport chain. Any of these steps could be rate-limiting, either by the activity of the involved enzymes, by the availability of competing substrates or by competition for cofactors such as CoA or NAD.

Previously published results have indicated that the fatty acid activation (Schultze *et al.* 1972, Flatmark and Pedersen 1975, Oram *et al.* 1973), the synthesis or translocation of acyl-carnitine, (Oram *et al.* 1973, Shephard *et al.* 1966, Fritz 1968, McGarry *et al.* 1973) the capacity of the β-oxidation cycle or the capacity of the electron transport chain (Pande 1971) might be the rate limiting step. Several of the involved reactions may vary under different experimental- or metabolic conditions in different organ systems from various

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Fig 1

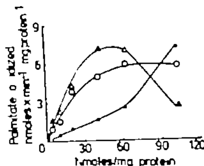


Fig. 2

1 The formation of 1-^{14}C-acetyl-groups from 1-^{14}C-palmitoyl-L-carnitine as function of the concentration of palmitoyl-L-carnitine. The incubation system contained in final volume of 300 µl 100 mM Tris-buffer pH 7.2, 4 mM dithiothreitol and 2.5 mM $MgCl_2$. Amount of mitochondrial protein was 1.2 … incubation temperature 35 and incubation time 2 min 1 ^{14}C-palmitoyl-L-carnitine was added as … Reaction was stopped by 150 µl 1.5 m KOH and BSA was added to concentration of 8 mg ml … from precipitate. 500 µl 4 M HClO was added, and after precipitation of the insoluble sub… an aliquot of the supernatant was transferred to counting vial containing 5 ml Unisolve ●—● 4 (no addition) △—△ State 3 (1 mM ADP 1 mM phosphate) ○—○ 4 µM CCCP (carbonyl … m-chlorophenyl hydrazone).

2 The -carnitine dependent formation of 1 ^{14}C-acetyl-groups from 1-^{14}C-palmitoyl-CoA function of the concentration of palmitoyl-CoA in the presence of 2.0 mM -carnitine. State 3 conditions were … by addition of 1 mM ADP 1 mM phosphate. Conditions otherwise were as stated in Legend … 1. ●—● Absence of -carnitine (no carnitine added) ○—○ State 4 (presence of -carnitine—absence … carnitine) — State 3 (presence of -carnitine—absence of -carnitine).

… were as stated in the Tables and Legends Figures. Aliquot of the perchloric acid-soluble … activity were counted in 5 ml Unisolve

The formation of 1-^{14}C-palmitoyl-CoA from 1 ^{14}C-palmitate CoA and ATP was assayed as indicated … 3, and 1-^{14}C-palmitoyl-CoA formed was extracted according to Bar Tana *et al* (1971)

… palmitoyl-CoA hydrolase was assayed by the release of 1 ^{14}C-palmitic acid from 1 ^{14}C-palmitoyl-CoA … previously described (Berge and Farstad 1978)

… respiratory control of the mitochondria (R C$_{ADP}$) was checked by the oxygen uptake with succinate … substrate in the absence and presence of ADP using Clark oxygen electrode (Radiometer Copenhagen, Denmark) (Gray *et al.* 1970)

Results

… formation of $^{14}CO_2$ from 1 ^{14}C-labelled substrates

Under the described conditions the formation of $^{14}CO_2$ was low during the first 1 2 min, … was linear with time up to about 6 min with all substrates (results not shown). In … experiments, the production of CO_2 was less than 4 per cent of the total acetyl-group formation provided the incubation time was 2 min or less. Thus, the error introduced by neglecting the CO_2 formation probably was within the over-all experimental error

Formation of ^{14}C-acetyl-groups from 1 ^{14}C-labelled palmitoyl-L-carnitine palmitoyl-CoA and palmitic acid

Fig. 1 shows the formation of ^{14}C-acetyl-groups from 1 ^{14}C-palmitoyl-L-carnitine as function of the palmitoyl-L-carnitine concentration. Saturation concentration was about … nmoles/mg protein under state 4 conditions. A half saturation concentration, or an

species (Oram *et al* 1973 Pande 1971 Bremer and Wojtczak 1972, Lumeng *et al* 197[illegible]). Fasting has been reported to have significant influence on fatty acid oxidation, although to a variable extent from species to species (Söling *et al* 1970), or no significant influence (DeMarco and Hoppel 1975)

Also an intramitochondrial long-chain acyl-CoA hydrolase recently described (Berge and Farstad 1978) is sufficiently active to influence the intramitochondrial acyl-CoA available for the β-oxidation cycle

The formation of acid soluble [14]C labelled oxidation products from [14]C-palmitoyl-L-carnitine has been shown to be a reliable method for the study of the β-oxidation (LaNoue *et al* 1970 Bremer and Wojtczak 1972, Oram *et al* 1973) The present communication is a study of factors that influence β-oxidation *in vitro*

Materials and methods

Reagent

1 [14]C palmitoyl L-carnitine (spec act 54 mCi/mmol), 1-[14]C-palmitoyl-CoA (spec. act. 56 mCi/mmol) and 1 [14]C-palmitic acid (spec act 52 mCi/mmol) were purchased from New England Nuclear, Boston, Ma U.S.A., palmitoyl-L-carnitine and L-carnitine from Supelco Inc. Bellefonte Pa U.S.A and other chemicals from Sigma Chemical Company St Louis, Mo. U.S.A

1 [14]C-labelled palmitoyl-carnitine and palmitoyl-CoA were dissolved in 30 per cent alcohol [illegible] concentration of 5 and 2.5 mM respectively Palmitate was used as a 5 mM aqueous solution of the potassium salt The specific activities of all substrates were about 500 000 counts/min per μmole [illegible] 5 ml Unisol e (Koch Light, Colnbrook Bucks, England) in a Packard Tri-Carb Scintillation Counter Model 2450.

Preparation of mitochondria

Male albino rats (Wistar Moll) of 150–200 g were killed and bled after fasting overnight The livers were immediately cut [illegible] pieces, cooled in ice and the mitochondria were isolated as described by Rosendal and Flatmark (1973). Protein was determined by a modified Lowry procedure (Flatmark *et al* 197[illegible]). The final mitochondrial suspensions contained about 50 mg protein per ml.

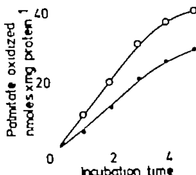
Preparation of mitochondrial matrix protein

Isolated mitochondria were treated with 0.12 mg digitonin per mg mitochondrial protein, and the matrix was isolated by centrifugation of the sonicated mitoplasts as described by Berge and Farstad (19[illegible]) The isolated matrix protein was virtually free from lysosomal and microsomal enzymes (Berge and Farstad 1978).

Assay methods

Production of $^{14}CO_2$ from [14]C-labelled substrates was assayed in 100 mM N 2-hydroxy-ethyl-piperazine-ethane-sulfonic acid (HEPES) pH 7.2 in Warburg flasks with 100 μl 1 M KOH in the center well and [illegible] μl 1 M H_2SO_4 in the side arm. The incubation medium contained 200 [illegible] mol of the radioactive substrate and 5 mg crystalline bovine serum albumin (BSA) per ml (about 40 nmol/mg protein). Incubation temperature was 35

The acid-soluble radioactivity from [14]C-labelled palmitoyl L-carnitine is a mixture of ketone bodies and Krebs cycle intermediates (LaNoue *et al* 1970), and thus is a measure of the β-oxidation through formation of acetyl-groups. The standard incubation for the formation of 1 [14]C-acetyl-groups from [14]C labelled substrates, which is a measure of palmitoyl-group utilization, was performed according to Bremer and Wojtczak (1972) with some modifications. 40–50 nmol of 1 [14]C-palmitoyl-CoA or palmitoyl-L-carnitine and 70–80 nmol of 1 [14]C-palmitate per mg mitochondrial protein were used. 100 mM HEPES buffer pH 7.2 was used if not otherwise stated. This buffer was chosen because it allowed low variation in toxicity without significant changes in oxidation of any of the substrates (see Results). Incubation temperature was 35 Incubation time 2 min, and amount of mitochondrial protein usually 1.2 mg/ml Of



The oxidation of palmitoyl-CoA as function of Conditions were as stated in Legend to Fig. 1 State 4 conditions (no additions). O—O State 3 conditions (ADP and phosphate)

ntrations of CoA (A), ATP (B), 1 ¹⁴C-palmitate (C) and L-carnitine (D). The oxida of palmitate was only moderately stimulated by exogenous CoA, and calculation of an rent K_m was not possible. It is certainly less than 5 μM and thus is in accordance with K_m for palmitoyl-CoA synthetase (Blom *et al.* 1976). The palmitate oxidation was not dependent on exogenous ATP (Fig. 3 B). Maximal palmitate oxidation was l at palmitate concentrations about 80–100 nmol/mg mitochondrial protein (Fig. 3 C) saturation concentration of approximately 30 nmol/mg protein (about 40 μM) is to the reported K_m for a "purified" palmitoyl-CoA synthetase (Bar Tana *et al.* 1971), very much higher than that reported by Pande *et al.* (1971). The optimal conditions for state oxidation are similar to previously reported optimal conditions for palmitate ation (Farstad and Sander 1971). Carnitine-dependence was found with concentrations 100 nmol palmitate/mg protein, then increasing carnitine independence occured (Fig.).

Initial rate measurements at concentrations from 0 to 20 nmol/mg protein of all substrates cated kinetics for palmitoyl-CoA and palmitoyl-carnitine similar to Michaelis-Menten ties, although slightly S-shaped curves were found at very low concentrations. Apparent values calculated from these experiments were about the same as calculated from strate saturation curves.

e effects of the incubation conditions on the oxidation of 1 C-palmitoyl-CoA 1-¹⁴C-palmitoyl-L-carnitine

4 show that with the incubation conditions stated, the formation of acetyl-groups from ¹⁴C-palmitoyl-CoA was linear with time up to about 3 min both under state 4 and state 3 ditions. Extending the incubation time over about 4 min also resulted in partial loss of nitine dependence.

Similar results were found when palmitoyl-L-carnitine was the substrate (not shown).

From data presented in Fig. 1–3 it seems justified to assume that substrate concentrations up to about 50 nmol/mg protein of palmitoyl-CoA and palmitoyl-L-carnitine, and up to about 80 nmol/mg protein of palmitate did not interfere with mitochondrial ability to oxidize palmitoyl-esters or palmitate under state 3 conditions, during the initial 3 min of incubation.

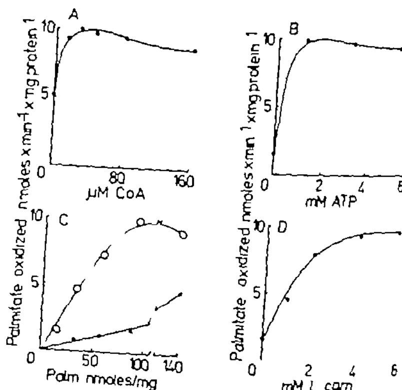


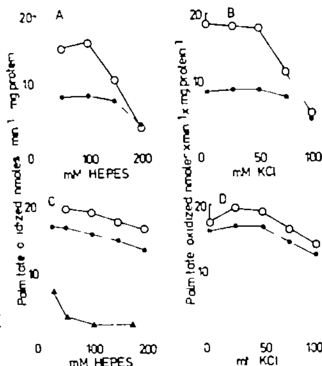
Fig. 3 The formation of 1-¹⁴C-acetyl-groups from 1-¹⁴C-palmitate as a function of the concentr[ation of] CoA (A), ATP (B), 1-¹⁴C-palmitat[e] (C) and L-carnitine (D). If not otherwise stated in the le[gend the] concentration of CoA was 50 μM, of ATP 2.5 mM, of 1-¹⁴C-palmitate 80 nmoles/mg protein an[d L-] carnitine 2.5 mM. In C ●—● represents absence of L-carnitine. Conditions otherwise as in le[gend to] Fig. 1.

apparent K_m for palmitoyl-L-carnitine was about 10 nmoles/mg protein. Both i[n the] presence of phosphate acceptor (state 3) and an uncoupler (CCCP), saturation conc[entra]tions were somewhat higher and the maximal oxidation rate was increased. In the fol[lowing] experiments 40–50 nmol palmitoyl-L-carnitine per mg protein were used.

Fig. 2 shows the L-carnitine dependent formation of acetyl-groups as a function o[f the] concentration of 1-¹⁴C-palmitoyl-CoA. At a concentration of L-carnitine of 1 mM, a [half-] saturation concentration, or an apparent K_m of 20 nmol palmitoyl-CoA/mg protei[n was] found. At a concentration of palmitoyl-CoA of 50 nmoles/mg protein the apparent [K_m] for L-carnitine was about 0.1 mM (not shown). As the kinetic for the first enzyme i[n the] oxidation of palmitoyl-CoA, carnitine palmitoyltransferase, is very complicated (Bremer [and] Norum 1967, 1967 a, 1967 b), a calculation of K_m values is, however, of limited value.

The concentrations of palmitoyl-CoA used (about 50 nmol/mg protein) do not seem [to] interfere with the formation of acid-soluble products from 1-¹⁴C-palmitoyl-CoA under s[tate] 3 conditions, although several other mitochondrial functions are known to be affe[cted] (Shrago *et al.* 1974, Davis and Lumeng 1974, Shug *et al.* 1971). Fig. 2 also shows that [the] oxidation of palmitoyl-CoA was dependent on exogenous L-carnitine up to about 60 n[mol/] mg protein. When palmitoyl-CoA was increased further, the β-oxidation became increasin[gly] independent of L-carnitine, probably due to increased mitochondrial permeability.

Fig. 3 shows the formation of acetyl-groups from 1-¹⁴C-palmitic [acid] as a function of t[he]



5 Oxidation of palmitoyl-L-carnitine as function of the concentration of HEPES (A) and KCl presence of 50 mM HEPES and the oxidation of palmitoyl-CoA as function of the concentration of HEPES (C) and KCl presence of 50 mM HEPES Conditions otherwise were as in Legend to Fig 1 and ●—● State 4 conditions additions) ○—○ State 3 (ADP and phosphate) ▲—▲ State 4 in the absence of -carnitine

the substrate When KCl was replaced by 200 mM sucrose in the presence of 50 mM HEPES oxidation rates were further decreased. Fig 5 furthermore shows that with the substrate concentration used the palmitoyl-CoA oxidation was dependent on exogenous L-carnitine even at 50 mM HEPES Addition of swelling agent (10 mM phosphate) to the standard incubation mixture increased the oxidation of palmitoyl-esters. The results indicate that the oxidation of palmitoyl-esters was inhibited in hypertonic media, probably due to dehydration of the matrix, in agreement with recently published effect of tonicity on oxidation of palmitoyl-L-carnitine (Osmundsen and Bremer 1976, Christiansen 1977 Nicholls et al 1972)

... of palmitoyl-CoA hydrolase from mitochondrial matrix

Recently we have shown (Berge and Farstad 1978) that liver mitochondria contain palmitoyl-CoA hydrolase localized to the inner membrane/matrix Digitonin fractionation of mitochondria removed almost completely microsomal and lysosomal contamination as the specific activities of microsomal marker and lysosomal marker were bout 1 1.5 per cent of the activity in whole homogenates. The specific activity of palmitoyl-CoA hydrolase in matrix as 160 per cent of that in intact mitochondria, and about 110 per cent of that in whole homogenate (Table II). The high hydrolase activity in the matrix suggests possible role in the intra-mitochondrial metabolism of long-chain acyl-CoA

TABLE I. The effect of malate, succinate and oxaloacetate on the oxidation of palmitoyl-L-carnitine and palmitoyl-CoA under state 4, state 3 and uncoupled conditions. Concentration of 1-^{14}C-palmitoyl-L-carnitine was 45 nmoles/mg protein, of 1-^{14}C-palmitoyl-CoA 50 nmoles/mg protein. Conditions otherwise as stated in Fig. 1-2. 1.2 mg mitochondrial protein were used. Concentrations of Krebs cycle intermediates were 5 mM, ADP 1 mM, phosphate 1 mM and CCCP 4 μM

Substrate	Nmoles radioactive product/mg protein/min		
	State 4	State 3 (+ADP/P)	Uncoupled
Palmitoyl-L-carnitine	7.8	11 7	10.2
malate	7 1	17.4	15 4
succinat	2.3	7 4	9.8
oxaloacetate	14 0	18.0	15 7
Palmitoyl-CoA	8 1	11 0	7 4
+ malate	10.8	15.2	10.0
succinate	5 1	8.8	8 1
+ oxaloacetat	16.2	20 0	11.5

Fig. 1 shows that the presence of CCCP stimulated palmitoyl-L-carnitine oxidation. Other experiments showed that uncoupling stimulated state 4 oxidation of palmitoyl-L-carnitine in freshly prepared mitochondria (R C$_{ADP}$ 5) whereas an uncoupler had no obvious effect with stored (RC$_{ADP}$ 3) mitochondria. The effect of CCCP with palmitoyl-CoA was more variable, CCCP rather inhibited palmitoyl-CoA oxidation with some mitochondria.

Swelling seriously interferes with several mitochondrial functions, although little loss of intramitochondrial nucleotides occurs during the first 2–3 min (Bremer and Wojtczak 19[illegible]; Bremer *et al* 1972). Weighing of the mitochondrial pellets indicated that under the present incubation conditions, the mitochondrial volume increased 15–20 per cent during the first ? min. The effect of swelling probably are of little importance, although Fig. 5 indicates that hypotonicity stimulated palmitoyl-CoA oxidation in our system.

The oxidation of palmitoyl L-carnitine was stimulated by oxaloacetate and inhibited by succinate both under state 3 and 4 conditions. Malate stimulated only under state 3 conditions (Table I). Addition of an uncoupler (CCCP) stimulated palmitoyl L-carnitine oxidation both in presence of malate and succinate, but not in the presence of oxaloacetate. Similar effects were observed with palmitoyl-CoA as the substrate both under state 3 and state 4. With this substrate, however, addition of CCCP partly reversed the effect of succinate and oxaloacetate.

Effect of tonicity on the oxidation of palmitoyl L-carnitine and palmitoyl-CoA

Fig. 5 shows that both palmitoyl L-carnitine and palmitoyl CoA were oxidized at a higher rate in a hypotonic medium than in an isotonic medium. Notably state 3 oxidation of palmitoyl L-carnitine was very high in hypotonic buffers. It is also seen that increased tonicity had a more pronounced effect with palmitoyl L-carnit[illegible] with palmitoyl-C[illegible]

III Observed values for β-oxidation of palmitate or palmitoyl-esters in relation to formation of palmitoyl-CoA and hydrolysis of palmitoyl-CoA by rat liver mitochondria. Data are extracted from Figures and Tables of this report and compared to results extracted from recent publications.

	Nmoles substrate/ min, mg protein		
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...ation of palmitoyl-CoA		11	
...ation of palmitoyl-L-carnitine		9	
...ation of palmitoyl-CoA		30	
...lysis of palmitoyl-CoA	9		12[a]
...toyl-CoA synthetase	70[b]		23[c]
...tine palmitoyltransferase	16[a]		
...ation of palmitoyl-L-carnitine	3.5[a]		
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[a] ...om Berge and Farstad (1978)
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[c] ...om Pande (1971) 28[a]
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TABLE II The activity of palmitoyl-CoA hydrolase in homogenate of mitochondria and isolated matrix in relation to a microsomal marker (NADPH cytochrome C reductase) and a lysosomal marker (acid phosphatase). Values in parentheses represent the specific activity as per cent of the specific activity in whole liver homogenate (For details see Berge and Farstad 1978)

	Specific activity (nmoles/min/mg protein)		
	NADPH cytochrome c reductase	Acid phosphatase	Palmitoyl-CoA hydrolase
Mitochondria	870	3.2	5.0
Isolated matrix	174 (1.5)	0.4 (1)	9.0 (110)

A possible rate limiting step in the oxidation of palmitate by rat liver mitochondria: Availability of palmitoyl CoA

From the data presented in Table I the maximally stimulated palmitoyl-ester oxidation was very high, up to about 70 nmol/min/mg protein in some expts. A possible rate limiting factor in the oxidation of palmitate in the intact cell is the availability of palmitoyl-CoA. Fig. 6 shows the formation of 1-14C-acetyl-groups and the formation of palmitoyl-CoA from 1-14C palmitate, CoA and ATP. During the first 2 min the formation of palmitoyl-CoA was higher than the oxidation of the palmitoyl-CoA formed. The initial rate of palmitoyl-CoA formation was about 25–30 nmoles per mg protein per min, but levelled off after about 4 min. Palmitate oxidation was low the first minute, then increased to about 10 nmol/min/mg protein. This was probably due to initial binding of palmitoyl-CoA to mitochondrial protein and insufficient palmitoyl-CoA was available for the carnitine palmitoyltransferase.

Addition of BSA resulted in a very strong inhibition of the oxidation of all substrates

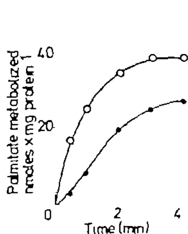


Fig. 7

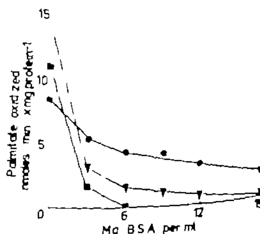


Fig. 7

Fig. 6. The formation of palmitoyl-CoA ○—○ and acetyl-groups ●—● from 1-14C-palmitate as a function of time by rat liver mitochondria (1.2 mg protein/ml). 1-14C-palmitoyl-CoA was extracted according to Bar Tana et al. (1971). Incubation conditions were as described in Legend to Fig. 3

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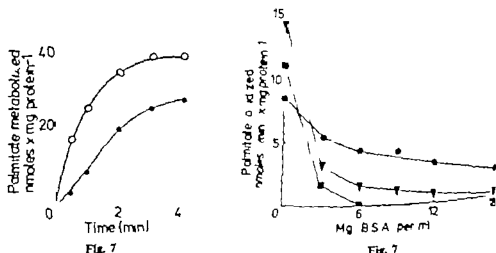


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Lumeng 1974) The *mitochondria* seemed, however functionally intact with respect of β-oxidation. *This* assumption is based on the following

1 β-Oxidation increased under state 3 conditions (ADP-stimulated).
2. β-Oxidation of palmitoyl-CoA was higher at concentrations independent of L-carnitine than at more *physiological* concentrations, indicating that hypotonicity increased β-oxidation
3 Uncoupling stimulated the *palmitoyl-ester* oxidation in fresh mitochondria, but not in aged mitochondria.

The *presence* of L-carnitine has a beneficial effect on the palmitoyl-CoA inhibition of the adenine nucleotide translocases (Shug *et al* 1971) and palmitoyl-CoA up to 20 nmol/mg protein has no effect *on* substrate level phosporylation, phosphate transport, as long as ATP-ases when incubation time is less than 5 min (Ho and Pande 1974). Also calcium-induced swelling *has little effect* on mitochondrial coupling (Hunter *et al.* 1976). Rat heart mitochondria oxidize palmitoyl-carnitine and palmitoyl-CoA optimally at 10–30 nmol/mg protein (McMillin Wood *et al* 1977) assayed by the oxygen consumption. The higher activities *obtained* by us are probably due a short incubation, as the effects of palmitoyl esters are time dependent (Ho and Pande 1974) Swelling of the mitochondria increased the β-oxidation in the *present* investigation, probably because of carnitine-independent transport of palmitoyl-CoA through the more permeable inner membrane (Fig. 2).

The effects of Krebs cycle *intermediates* are in agreement with well-known observations that changes in the NADH NAD ratio and competition at the flavoprotein step in the respiratory chain are major *regulatory* steps in fatty acid oxidation and ketogenesis (Bremer and Wojtczak 1972, Lumeng *et al* 1976 Lopez-Cardozo and van den Bergh 1972, Varge *et al* 1972) The finding that succinate was less inhibitory with palmitate or palmitoyl-CoA as substrates than with *palmitoyl*-L-carnitine as substrate may however indicate that the respiratory chain will not be the rate limiting step under physiological conditions.

The present results indicate that the *β-oxidation* cycle is less sensitive to changes in mitochondrial integrity than the Krebs cycle linked respiration (Pande 1971 McMillin Wood *et al* 1977), and that state 3 respiration can be obtained at very high substrate concentrations in agreement with Rafael and Wrabetz (1976)

From the present results it seems warranted to conclude that all steps involved in fatty acid activation translocation over the *inner* mitochondrial membrane, *β-oxidation* and respiratory chain transport are *sufficiently* active to meet all fatty acid oxidation taking place in the intact cell In the cytosolic space of rat liver the calculated acyl-CoA probably never exceeds 1 nmol/mg protein *according* to available data (Nordlie *et al* 1967). Thus it seems likely that the availability of long-chain acyl-CoA will be the dominating regulatory factor of fatty acid oxidation in the intact rat liver cell. On the *other* hand the capacity of β-oxidation in the present study is in accordance with the *β-oxidation* in intact hepatocytes calculated to be up to 8–10 nmol/min/mg mitochondrial protein under stimulated conditions (Christiansen 1977)

The authors are gratefull to the excellent assistance of cand. mag. Bjorg Dowland. The study was supported by grants from the Norwegian *Council of* Cardiovascular Disease and the Norwegian Council of Science and the Humanities.

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Effects of dopaminergic agonists and antagonists on isolated cerebral blood vessels

By

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Abstract

Edvinsson, L., J. E. Hardebo, J. McCulloch and Ch. Owman. *Effects of dopaminergic agonists and antagonists on isolated cerebral blood vessels.* Acta physiol. scand. 1978. *104.* 349–359.

The vasomotor responses of dopamine and dopaminergic agonists was studied in vitro on middle cerebral arteries from cat and pial arteries from humans. The action of various inhibitors was tested in order to define the receptors involved. A contractile response could be obtained by epinine, apomorphine and dopamine in the mentioned order of potency. The effect was blocked by alpha-receptor as well as serotonin receptor antagonists. The mode of inhibition suggested that serotonin receptors rather than alpha-adrenoceptors mediated the dopamine-induced contraction. A dose-dependent dilatation could be evoked by the dopaminergic agonists on actively contracted pial arteries. The relative potency was epinine > dopamine > apomorphine. The order of potency for the agonists, together with blocking experiments (including parallel shift in the log dose-response curve induced by bulbocapnine), indicated that the vasodilatation is mediated by specific dopamine receptors.

Ever since it became clear on the basis of chemical determinations that dopamine has a regional distribution within the brain (Bertler and Rosengren 1959, Bertler 1961) and that it was established that the catecholamine was localized within the neurons in the central nervous system (Carlsson, Falck and Hillarp 1962), numerous studies have been carried out to describe the organization and function of the central dopaminergic systems. As the histochemical techniques have been improved in sensitivity and specificity, many dopamine systems, which were previously undetected, have been disclosed recently. The central dopamine projection systems which have been characterized at the present time (see Lindvall and Björklund 1977 for review) are the mesostriatal, the mesocortical, the periventricular, the incertohypothalamic, and the tuberohypophyseal systems as well as the periglomerular dopamine neurons.

These widespread dopaminergic systems are closely associated with microvessels within the brain. Although dopaminergic neurons have not, as yet, been shown to terminate directly on cerebral blood vessels, this type of arrangement has been proposed to occur in regions such

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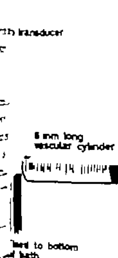


Fig. 1

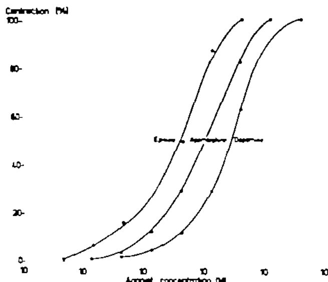


Fig. 2

Fig. 1. Arrangement for suspension of vascular cylinder between two L-formed metal prongs for recording of circular contractile activity

Fig. 2. Contraction in vitro of cat's middle cerebral artery in response to epanine, apomorphine, and dopamine to show relative agonist potency in a representative experiment.

concentrations below are given as the salt and expressed as the final molar concentration in the buffer solution of the organ bath

Analysis of data. Concentration-response curves were obtained by cumulative application of the drugs to the organ bath. The irreversible antagonist, phenoxybenzamine, was present for 30 min in the organ bath followed by several washouts before the agonists were tested again. The reversible inhibitory drugs phentolamine, piperoxane, cyproheptadine, methysergide, haloperidol, bulbocapnine and propranolol were added 30 min before and were present during the tests. In cases in which the antagonism of the response involved a decrease in the maximum and the slope of the actual log dose-response curves, the E_{Am} of the curve before addition of the antagonist was set as 100 and the subsequent curves obtained after blockade were related to this value. When antagonism only involved a parallel shift in the actual log dose-response curves, the E_{Am} of each curve from the experiment was set as 100 %. The response was also characterized with regard to the concentration of the drug resulting in half-maximum response (ED_{50}). The apparent dissociation constant for the receptor-antagonist complex (K_B) was calculated according to the equation $[A']/[A]-1=[B]/K_B$, where $[A']/[A]$ (= dose ratio) is the ratio of concentrations of agonist giving an equal response in the presence and in the absence of a given concentration of the antagonist, [B] (Furchgott 1972). Calculation of differences between mean values were carried out with a Hewlett-Packard desk computer.

Results

Contractile response

The preparation used (feline middle cerebral artery and small human pial arteries) showed no spontaneous contractions. Under resting conditions, i.e. when the vessels had reached a steady level of tension (approximately 300 dyn), epinine, apomorphine and dopamine all produced a contractile response. The effect fairly rapidly reached a plateau, which was main-

as the caudate-putamen (Hartman, Zide and Udenfriend 1972). It has recently been suggested that in addition to any direct vasomotor influence dopamine may exert upon cerebral vessels, the manipulation of intracerebral dopaminergic systems may alter cerebral tissue perfusion indirectly, namely by changing the metabolic needs of the brain (McCulloch and Harper 1977).

Specific dopamine receptors have been suggested to occur in renal, mesenteric and coronary vessels but not in the femoral and carotid vasculature (see review by Goldberg 1972). Recent studies have demonstrated that dopamine is able to both contract and dilate cerebral blood vessels *in vitro* (Toda *et al.* 1975, Toda 1976, Edvinsson *et al.* 1977). This has been confirmed under *in vivo* conditions using the microapplication technique where dopamine was found to constrict pial arterioles on the surface of the cerebral cortex of cats and apomorphine, a dopamine receptor antagonist, dilated these vessels (Edvinsson *et al.* 1977). Little is, however, known about the detailed receptor mechanisms mediating this dopamine action on the cerebrovascular bed. In order to define the nature of the receptors involved, dopaminergic agonists and antagonists have been tested on segments of cerebral vessels *in vitro*.

Material and methods

Animals. Specimens of the middle cerebral artery were obtained from 25 adult cats of either sex, weighing between 1.8 and 4.5 kg. All animals were killed by bleeding under sodium pentobarbital anesthesia, the brain was removed, and the pial vessels were immediately dissected out and placed in aerated Krebs-Ringer buffer solution (for composition, see below). Part of the material was immediately used in the experiments, whereas the rest was used after storage in the buffer solution in a refrigerator (+4°C) for up to 24 h.

Denervation. Sympathectomy was carried out under sodium pentobarbital anesthesia (30 mg/kg i.p.) in 5 of the animals by unilateral or bilateral excision of the superior cervical ganglia at least 1 week prior to the experimentation. These preliminary tests did not demonstrate any significant difference between the two types of vessels with regard to their response to dopaminergic agonists. Therefore the majority of experiments were carried out on innervated middle cerebral arteries.

Human material. During lobe resection in conjunction with neurosurgical tumor operations, small segments of human pial arteries on the surface of the temporal and parietal lobes were dissected free, immersed into ice-cold Krebs-Ringer buffer solution and immediately transported (15 min) to the laboratory and mounted as above for registration of vasomotor activity.

Recording of vasomotor effects. Small segments of the middle cerebral artery of cats, about 300-400 µm wide and 5 mm long (Fig. 1), and small surface pial arterioles of similar size from humans were suspended in an organ bath containing 50 ml of bicarbonate-buffered Krebs-Ringer solution. The solution was aerated continuously with 95% O_2–5% CO_2 mixture and maintained at 37°C. The composition of the Krebs-Ringer solution (millimoles per liter) was: NaCl 118, KCl 4.5, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \times 7H_2O$ 1.1, KH_2PO_4 1.0, $NaHCO_3$ 25 and glucose 6.0. Washout of the chamber was made by renewing its total content. The segments were suspended between two L-formed metal prongs attached to Endevco Model 8107 isometric force-displacement transducers for recording of circular contractile activity on a Grass Model 7 B polygraph. The basal tension on each vessel segment (2 segments were suspended in the same bath) was adjusted to approximately 400 dyn after which it was allowed to relax and accommodate for 2 h prior to the addition of any drug (Edvinsson, Nielsen and Owman 1974, Edvinsson and Owman 1974).

Drugs. The following compounds were used: Apomorphine hydrochloride (ACO, Sweden), L-arterenol hydrochloride (Sigma), epinine hydrochloride (Regis), dopamine hydrochloride (Nutritional Biochem. Corp., USA), 5-hydroxytryptamine creatinine sulfate (Sigma), DL-isoproterenol hydrochloride (Sigma), bulbocapnine (Smith, Kline and French), cyproheptadine (Merck, Sharp and Dohme), haloperidol (AB Leo, Sweden), methysergide hydrogenmaleinate (Sandoz), phenoxybenzamine hydrochloride (ACO, Sweden), phentolamine methansulphon (Hässle, Ciba, Geigy), pimozide (AB Leo, Sweden), piperoxane hydrochloride (Rhône Poulenc), propranolol hydrochloride (Draco, Sweden).

All drugs were dissolved in 0.9% saline (bulbocapnine and pimozide after adding a few µl of 1 M HCl).

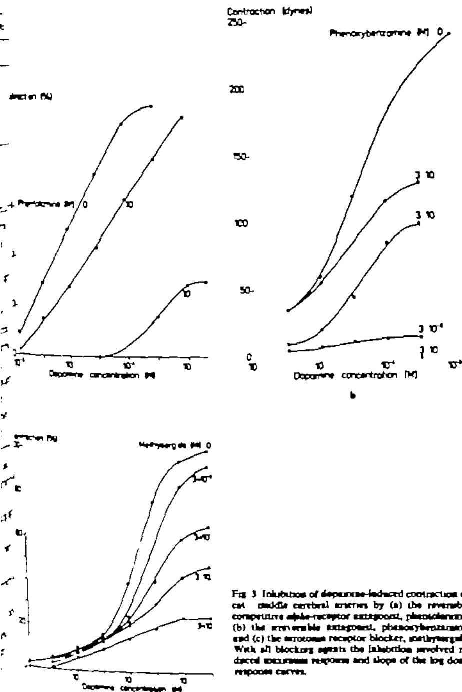


Fig. 3. Inhibition of dopamine-induced contraction of cat middle cerebral arteries by (a) the reversible competitive alpha-receptor antagonist, phentolamine (b) the irreversible antagonist, phenoxybenzamine, and (c) the serotonin receptor blocker, methysergide. With all blocking agents the inhibition involved reduced maximum response and slope of the log dose-response curves.

TABLE I Contractile response of feline isolated middle cerebral arteries to dopaminergic compounds in comparison with norepinephrine and 5-hydroxytryptamine. Mean values ± S.E.

Agonist	Number of tests	ED_{50} values (M)	E_{max} values (dyn)
Dopamine	14	$(3.80 \pm 1.41) \times 10^{-6}$	327 ± 55
Epinine	4	$(6.17 \pm 1.29) \times 10^{-6}$	227 ± 56
Apomorphine	7	$(1.00 \pm 1.29) \times 10^{-6}$	83 ± 19
Norepinephrine	10	$(2.17 \pm 0.55) \times 10^{-6}$	233 ± 46
5-Hydroxytryptamine	15	$(3.45 \pm 1.01) \times 10^{-6}$	304 ± 46

tained long enough to allow for cumulative application of increasing doses. After rinsing the arteries returned to their initial level of tension.

In some instances the experiments were also performed on sympathectomized arteries from cat (1 week postoperatively). No difference was seen in the response between vessels from operated and non-operated animals. The same pattern of response under the various conditions given below was noted in preparations from both feline and human vessels.

Relative potency. In order to evaluate the relative potency of the agents mentioned above with regard to their contractile effects, full dose-response curves were constructed, and the mean concentrations required to obtain half-maximum contraction (ED_{50}) were compared (Fig. 2). The relative potency was epinine > apomorphine > dopamine, as summarized in Table I. However, dopamine produced the strongest contractile response, which was of the same order of magnitude as that produced by 5-hydroxytryptamine (Table I).

Competitive antagonism. If the contractile effect of the dopaminergic agents is mediated by *alpha*-adrenergic receptors, reversible competitive antagonists, such as phentolamine and piperoxan, should decrease the sensitivity of the test system to the agonists without decreasing the maximum or the slope of the log dose-response curve (Furchgott 1972). This was tested by using dopamine as the agonist. It was found that both phentolamine (Fig. 3 a) and piperoxan in increasing concentrations caused a decrease in the slope and the maximum of the dose-response curves, suggesting that the inhibitory effect on the dopamine-induced contraction was not mediated specifically by *alpha*-adrenergic receptors.

The β-haloalkylamine, phenoxybenzamine, is a competitive blocking agent which, for practical purpose, can be considered as irreversible through its ability to inactivate the receptor sites (Furchgott 1972). In the present study, increasing doses of phenoxybenzamine caused a dose-dependent reduction in the slope and in the maximum contractile effect (Fig. 3 b). A dose of $3\text{–}5 \times 10^{-6}$ M phenoxybenzamine was required to completely abolish the dopamine induced contraction (Fig. 3 b).

The two serotonin receptor antagonists, methysergide (Fig. 3 c) and cyproheptadine, also inhibited the dopamine induced contraction by reducing the maximum and the slope of the dose-response curves in the same way as previously found with 5-hydroxytryptamine as agonist in cerebral blood vessels (Edvinsson, Hardebo and Owman 1978).

Dilatory response

In studies on the dilatory effect of dopaminergic agonists, the pial arteries were exposed to $3\text{–}5 \times 10^{-6}$ M phenoxybenzamine for 30 min to block their contractile effects. Furthermore,

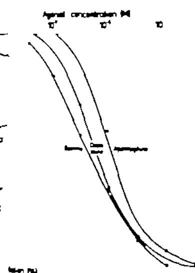


Fig. 5

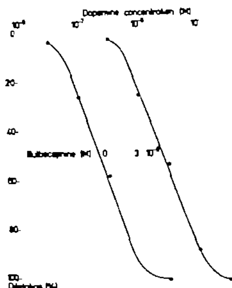


Fig. 6

Fig. 5. Relative dilatory effect of cat's middle cerebral artery obtained by epinine, dopamine, and apomorphine (after blocking contraction by exposure to 3-5 10^{-6} M phenoxybenzamine for 30 min and after bringing the vessel to active tone by prostaglandin $F_{2\alpha}$).

Fig. 6. Parallel shift of dilatory log dose-response curve for dopamine in the presence of bulbocapnine. Conditions as in Fig. 5.

...e vessels to dopamine (and in a few experiments apomorphine) was reduced by administration of increasing concentrations of bulbocapnine (Fig. 6). The apparent K_B value was ...und to be (1.65 ± 0.51) 10^{-6} M from 10 tests. However in high concentrations (above ...10^{-5} M) this antagonist caused a vascular dilatation of its own.

The reversible *beta*-blocking agent, propranolol, known to cause a marked parallel shift ...f the dose-response curve to isoproterenol (Edvinsson and Owman 1974) was without ...ffect on the dopamine-induced relaxation.

Discussion

Dopamine has complex vascular effects and may both contract and relax blood vessels (Cohen and Berkowitz 1975). Attempts to elucidate the mechanism(s) for the effects produced by dopamine have included reports of receptors specific to dopamine in ganglia (Kebabian and Greengard 1971), brain (Kebabian, Petzold and Greengard 1972) and in arteries within mesenteric, renal (McNay and Goldberg 1966, Yeh, McNay and Goldberg 1969, Higgins *et al.* 1973) and coronary (Schuelke *et al.* 1971) vascular beds. Apomorphine has been used as a tool to confirm the presence of dopamine receptors since it has been shown to possess structural (Goldberg, Sonneville and McNay 1968), biochemical and functional (Andén *et al.* 1967) effects similar to dopamine.

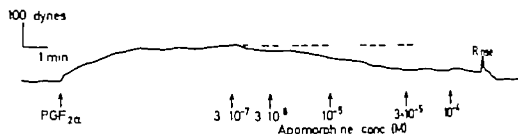


Fig. 4 Representative experiments showing tonic contraction induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the dilatory effect of apomorphine in increasing concentrations. The dashed line indicates the steady level of contraction in the presence of $PGF_{2\alpha}$ alone

in order to reveal a clear-cut dilatory response the arteries were given an active tonic contraction by adding 2.5 10⁻ M prostaglandin $F_{2\alpha}$. This caused in the present series of experiments a strong contraction amounting 560 ± 92 (mean ± S.E.) dyn, which remained at a steady level for more than 20 min *i.e.* long enough to allow for cumulative application of various agents (Fig. 4)

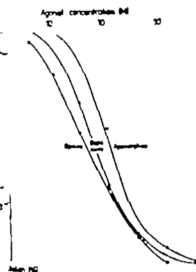
Relative potency The ability to produce a dilatation (relaxation) of the feline middle cerebral artery and human pial arteries was tested under the conditions described above (Fig. 4) The relative potency (comparison of mean ED_{50} values) was found to be epinine dopamine > apomorphine (Table II) The effects were dose-dependent and amounted 30-90 dyn (Fig. 5) For comparison, isoproterenol dilated the preparation by about 120 dyn, and the ED_{50} values were lower than for the dopaminergic agonists. Relaxations by dopamine and dopaminergic agents were obtained in about 50 % of the experiments. In others either no effect or a contraction was obtained If a further dose of phenoxybenzamine was applied the contraction could be abolished and sometimes a dilatation could be demonstrated.

Competitive antagonism Experiments were undertaken with several putative dopamine receptor antagonists to reveal the characteristics of the receptor involved in this dopamine-induced dilatation Low concentrations of haloperidol (less than 10⁻⁶ M) did not shift the dopamine-induced dilatation towards higher doses. Greater concentrations of haloperidol (*i.e.* concentrations at which the drug acts as a dopamine antagonist at other sites) dilated the preparation to the same degree as dopamine and in the concentrations supposed to be antagonistic. Pimozide, another dopamine receptor antagonist, could not be dissolved in the buffer solution used.

Bulbocapnine, which has recently been suggested to act as a specific dopamine receptor antagonist, was able to alter the dopamine curve towards higher doses, *i.e.* the sensitivity of

TABLE II Dilatation of the middle cerebral artery after blockade with 3 10⁻⁶ M phenoxybenzamine and after giving the vessels an active tonic contraction amounting 560 ± 92 dynes by prostaglandin $F_{2\alpha}$ (2.5 10⁻⁶) Results are expressed as mean values ± S.E.

Agonist	Number of tests	ED_{50} values (M)	E_{Am} values (dynes)
Dopamine	13	(8.45 ± 1.56) 10⁻	92 ± 20
Epinine	6	(3.83 ± 1.21) 10⁻	31 ± 4
Apomorphine	5	(9.42 ± 3.21) 10⁻⁶	90 ± 19
Isoproterenol	10	(1.26 ± 0.63) 10⁻⁶	116 ± 20



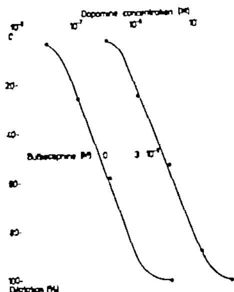


Fig. 5 Fig. 6

5. Relative dilatory effect of cat's middle cerebral artery obtained by epinine, dopamine, and apomorphine (after blocking contraction by exposure to 3–5 10^{-6} M phenoxybenzamine for 30 min and after bringing the vessel to active tone by prostaglandin $F_{2\alpha}$).

6. Parallel shift of dilatory log dose-response curve for dopamine in the presence of bulbocapnine. Conditions as in Fig. 5.

...e vessels to dopamine (and in a few experiments apomorphine) was reduced by administration of increasing concentrations of bulbocapnine (Fig. 6). The apparent K_B value was found to be (1.65 ± 0.51) 10^{-4} M from 10 tests. However in high concentrations (above 10^{-4} M) this antagonist caused a vascular dilatation of its own.

The reversible *beta*-blocking agent, propranolol, known to cause a marked parallel shift of the dose-response curve to isoproterenol (Edvinsson and Owman 1974) was without effect on the dopamine-induced relaxation.

Discussion

Dopamine has complex vascular effects and may both contract and relax blood vessels (Cohen and Berkowitz 1975). Attempts to elucidate the mechanism(s) for the effects produced by dopamine have included reports of receptors specific to dopamine in ganglia (Kebabian and Greengard 1971), brain (Kebabian, Petzold and Greengard 1972) and in arteries within mesenteric, renal (McNay and Goldberg 1966, Yeh, McNay and Goldberg 1969, Higgins *et al.* 1973) and coronary (Schuelke *et al.* 1971) vascular beds. Apomorphine has been used as a tool to confirm the presence of dopamine receptors since it has been shown to possess structural (Goldberg, Sonneville and McNay 1968), biochemical and functional (Andén *et al.* 1967) effects similar to dopamine.

Contractile response

Dopamine, apomorphine and epinine all constricted the feline middle cerebral artery in a dose-dependent way at relatively high doses. The contractile effect of dopamine could be attenuated by relatively high concentrations of the adrenergic blocking agents phentolamine, piperoxan and phenoxybenzamine. Dopamine induced contractions have been demonstrated in peripheral arteries and veins, both in isolated vessels and in intact vascular beds. These responses are due to the activation of *alpha*-adrenergic receptors (*cf.* Goldberg 1975). In rabbit aorta the contraction produced both by apomorphine and dopamine could be blocked by dibenamine, suggesting that both agonists contract this vascular smooth musculature by a similar *alpha*-adrenergic receptor stimulation (Cohen and Berkowitz 1976). Previous work on cranial vessels has shown a constrictor response following administration of dopamine to the isolated middle cerebral and basilar arteries (Toda 1976), and a vasoconstriction has also been revealed under *in vivo* conditions (White, Hagen and Robertson 1976; Edvinsson *et al.* 1977). This constrictor response was abolished by the *alpha*-receptor blocking agents. During infusion of small doses of dopamine (< 1 mg/kg/min h) to dogs, the cerebral blood flow is reduced, which has been ascribed to an effect on *alpha*-adrenergic receptors (von Essen 1974). In the present study it was found that the competitive reversible *alpha*-receptor antagonist, phentolamine and piperoxan, did not cause a parallel shift of the log dose-response relation of dopamine but rather reduced the slope and maximum effect, which is not consistent with a clear-cut action of dopamine on *alpha*-adrenergic receptors. In other studies, a dose of 3 × 10^{-4} M of phenoxybenzamine was needed to totally block a norepinephrine induced contraction (Edvinsson and Owman 1974). This concentration reduced the response to serotonin but did not abolish it (Edvinsson, Hardebo and Owman 1978). Since a concentration of 3–5 10 M phenoxybenzamine was required to abolish the dopamine induced contraction, the findings would indicate that the dopamine induced cerebral vasoconstriction is mediated at least in part by other receptors than *alpha*-adrenergic. A similar conclusion was reached by Gilbert and Goldberg (1975) in experiments on canine femoral and carotid arteries. They observed that the 5-hydroxytryptamine receptor blocking agent cyproheptadine inhibited contractions induced by dopamine, tryptamine and 5-hydroxytryptamine in both types of arteries to a similar extent, whereas the norepinephrine induced contractions were unaffected. Protection experiments suggested that the vasoconstriction evoked by dopamine is to a large extent mediated by serotonin receptors. The same mechanism may hold true also for the brain vessels, where cyproheptadine and methysergide were able to inhibit both dopamine- and 5-hydroxytryptamine-induced contractions, without affecting the contractile effect of norepinephrine (Edvinsson *et al.* 1978). The relatively high doses of phenoxybenzamine required to block the dopamine response is consistent with the dose level at which the 5-hydroxytryptamine-induced contraction of pial arteries is blocked.

Dilatory response

In addition to contraction of the pial vessels, relaxation can be observed following phenoxybenzamine blockade. After this treatment neither serotonin nor *alpha*-adrenergic

ptor agonists were capable of producing a satisfactory contraction, and therefore staglandin $F_{2\alpha}$ was used to induce the active contractile tension in these preparations. the pial arteries dilated (relaxed) upon administration of the dopaminergic compounds in following order of potency: epinine > dopamine > apomorphine. It has been suggested experiments on aortic strips that the effect of dopamine is mediated by an increase in c AMP levels (Cohen and Berkowitz 1975). In accordance with this, administration of c AMP has been found to produce a dose-dependent relaxation of pial artery prepara- s (Hardebo and Edvinsson 1979). Apomorphine, in concentration that produced the e degree of relaxation as dopamine, was less effective in altering cyclic AMP levels hen and Berkowitz 1975). This is in accordance with the present study where dopamine found to be more effective than apomorphine in dilating the pial vessels. When the a-receptors are blocked, dose-related vasodilations have been shown to occur in the al and mesenteric vascular beds after intra-arterial injections of dopamine (Goldberg 2) and there is some evidence that, within the cerebral vascular bed, blood flow may also increased in similar circumstances (von Essen 1974).

Dopamine increases cardiac contractility and heart rate (Goldberg 1975): these actions are agonized by propranolol, indicating that they are due to stimulation of cardiac *beta*-eptors. On the other hand, dilatation of the pial arteries caused by dopaminergic agents s not affected by propranolol in concentrations sufficient to cause a marked shift in the e-response curve of isoproterenol. Similar findings have been obtained from studies on lated renal, mesenteric, coronary and femoral arteries. Moreover this vasodilatation was t affected by atropine, antihistaminics, or by pretreatment with reserpine, compound 80, or monoamine oxidase inhibitors (see Goldberg 1972, 1975).

There is reason to assume that the dopaminergic agents act on a specific dopaminergic ceptor to produce dilatation in peripheral arteries. In the present study attempts to de- itively characterize the receptor by which dopamine dilates cerebral arteries have been ly partially successful, although a number of pieces of circumstantial evidence point to it ing specific dopamine receptor. The dopamine-induced relaxation of renal and mesen- ric vessels has been antagonized selectively by haloperidol (Goldberg 1972). In the present udy this was not possible to achieve since haloperidol itself relaxed the pial arteries in doses quired to inhibit the vasodilatation produced by dopamine. On the other hand however lbocapnine, a peripheral dopamine receptor antagonist, shifted the dose-response curve of opamine in isolated pial arteries towards higher doses. Bulbocapnine has a structural milarity with dopamine and apomorphine and has been shown to antagonize peripheral ascular dopamine effects, probably in a competitive way (Pendleton, Finlay and Sherman 975; Setler, Pendleton and Finlay 1975). Bulbocapnine possesses some *alpha*-adrenolytic Van Rossum 1965), but no anticholinergic, antihistaminic, or *beta*-adrenolytic activity. However, bulbocapnine, in doses above $1 \cdot 10^{-4}$ M exerts a dilatory effect on isolated artery egments. It is not unlikely that this dilatation is the result of a dopamine agonistic property of bulbocapnine.

Recent investigation have demonstrated that the administration of apomorphine results in ncreases in cerebral blood flow accompanied by an elevated cerebral oxygen and glucose consumption (McCulloch and Harper 1977). It was suggested that the primary action of

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apomorphine was to stimulate cerebral metabolic activity and that the increased cerebral tissue perfusion was secondary to this. The preliminary observations (Edvinsson et al. 1977) of apomorphine-induced dilatations of the pial arterioles on the convexity of the brain following perivascular microapplication, together with the *in vitro* experiments, suggest that a direct action of apomorphine upon the cerebral vasculature may contribute to the increase in cerebral tissue perfusion noted with this agent (McCulloch, Teasdale and Harper 1977). The observations of dopamine-induced changes in cerebral blood flow by von Essen (1974), although subject to methodological criticism, could be considered to be consistent with both the observations *in vitro* and *in vivo* of the present study.

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A definition of the extraction. We consider an experimental situation in which a number of molecules (ions), *n*, is injected as a bolus into the artery of an organ. After injection at time $t = 0$, the number of injected molecules can be divided according to their history at time *t*, such that

$$n = N_E(t) + N_T(t) + N(t) \quad (1)$$

where $N_E(t)$ is the number of molecules that have been extracted (i.e. passed the capillary membrane at least once) at time *t*, $N_T(t)$ is the number of molecules that have been transmitted (i.e. passed the capillaries without having been extracted) at time *t* and N(t) is the number of molecules that have been neither extracted nor transmitted at time *t*

There exists a time $t_s > 0$, such that

$$n = N_E(t) + N_T(t) \quad (2)$$

The extraction is

$$E = N_E(t)/n, \text{ for } t > t_s \quad (3)$$

Conservation of test molecules. We assume that the number of injected molecules can be measured in a fixed part of the organ by external detection. The detector signal can be expressed as

$$S(t) = n - N_w(t) \quad (4)$$

and

$$S(t) = N_a(t) \quad N_c(t) + N_{ev}(t) + N_v(t) \quad (5)$$

for all $t \geq t'$ where t' is the time when the last molecule in the bolus has entered the detector field. The subscript denotes the molecules present in the venous blood outside of the detector field and the subscripts *a*, c, *ev* and denote the molecules in the arteries, the capillaries, the extravascular space and the veins in the detector field, respectively

The number of molecules that has traversed the capillary membrane at least once at time *t* can be expressed in terms of their position as

$$N_E(t) = N_{ev}(t) + N_{E,c}(t) + N_{E,v}(t) + N_{E,w}(t) \quad (6)$$

Similarly the molecules that have been transmitted at time *t* can be expressed in terms of their position as

$$N_T(t) = N_v(t) - N_{E,v}(t) + N_w(t) - N_{E,w}(t) \quad (7)$$

where

$$N_w(t) = N_{E,w}(t) + N_{T,w}(t) \quad (8)$$

At time $t > t_w$ we have that

$$N_a(t) = 0 \quad (9)$$

$$N_c(t) = N_{E,c}(t) \quad (10)$$

A proposition. According to Sejrsen (1970), the function

$$F(t) = N_w(t) \quad (11)$$

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A note on the single injection residue function method to determine capillary permeability

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Abstract

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The assumptions inherent to the single injection residue function method to determine capillary permeability have been made explicit Furthermore it is shown that agreement between the deduced wash-out function of the transmitted molecules of the test substances and that of reference molecules does not constitute proof of the validity of the method

Key words: Capillary permeability theory of measurements of capillary permeability

Measurements of capillary solute extraction can be used to estimate capillary permeability Crone (1961) has proposed that the extraction obtained as the relative difference between the initial areas of the concentration-versus-time curves in the venous blood following injection of a bolus containing the test molecule and reference molecule can be given a simple interpretation of capillary diffusion capacity Problems related to the measurement and interpretation of this extraction, *i.e* the single injection venous up-slope (SIVUS) method, have been discussed (see Crone and Lassen 1970)

Sejrsen (1970) proposed an alternative method of measuring the extraction. It is based on external measurement of the residue function and thus has the interesting feature that it can be used on organs with multiple outlets. Furthermore, the method is much less demanding from an experimental point of view and therefore of considerable potential clinical interest. The method, *i.e* the single injection residue function (SIRF) method has recently been applied to adipose and subcutaneous tissue (Paaske and Levin Nielsen 1976, Paaske 1976, Paaske 1977 a and b) However Sejrsen did not offer any theoretical justification for the method and it is not obvious what kinds of assumptions that are involved over and above those used in the SIVUS method

The present paper is an attempt to analyze the situation in some more detail with the object of making explicit the assumptions inherent to the SIRF method

It has been proposed (Paaske and Levin Nielsen 1976, Paaske 1976, Paaske 1977) that the validity of the SIRF method can be substantiated by the experimental finding that the deduced wash-out function for the transmitted molecules is identical to the observed wash-out function for a reference molecule that cannot pass the capillary membrane, *i.e.* that

$$\frac{S(t) - F(t)}{S(t_1) - F(t_1)} \quad \text{is numerically equal to} \quad \frac{S'(t)}{n} \tag{21}$$

where $S'(t)$ is the *detector signal function* for the reference molecules and n is the number of injected reference molecules. We show below that this observation cannot be considered as a strong proof of the validity of the SIRF method.

For any time $t > t$ t it must be true that

$$\frac{S'(t)}{n'} = \frac{N_T(t_s)}{} \frac{N_p(t) + N_{E,v}(t)}{N_T(t_s)} \tag{22}$$

However using (5) and (7)

$$\frac{S(t) \quad F(t)}{S(t_1) \quad F(t_1)} \quad \frac{N_s(t) \quad N_p(t) \quad N_T(t) + N_{E,v}(t) \quad N_s(t) + N_{E,v}(t) \quad N_{ev}(t) \quad F(t)}{N_s(t_1) \quad N_s(t_1) + N_T(t_1) + N_{E,v}(t_1) - N_s(t_1) + N_{E,v}(t_1) + N_{ev}(t_1) \quad F(t_1)} \tag{23}$$

The right hand sides of (22) and (23) become identical when

$$t > t_s \tag{24}$$

$$N_{E,}(t) = N_{E,v}(t) = 0 \tag{25}$$

$$N_s(t_1) = 0 \tag{26}$$

$$F(t) \quad N_{ev}(t) \tag{27}$$

Thus, if the assumptions are correct, there will be numerical identity between the deduced wash-out curve for the transmitted molecules and the observed wash-out curve for the reference molecules. However as shown by the expressions (22) and (23), an observed numerical identity does not permit the conclusion that the assumptions are correct.

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can be determined for all values of $t > t_1$ where t is the time at which S(t) has its maximum value. The proposition is based on the following two assumptions

(i) There exists a time $t = t > t$ when all the transmitted molecules have left the detector field. Thus,

$$N(t) = 0 \quad \text{for } t > t_s \tag{12}$$

It is assumed that

$$N_s(t) \sim N_s(t) < N_{ev}(t) \quad \text{for } t > t_s \tag{13}$$

so that according to (5)

$$N_{ev}(t) \sim S(t), \text{ for } t > t \tag{14}$$

(ii) The form of S(t) for $t > t_s$ can be used, by means of extrapolation back to $t = t_1$ to determine $F(t_1) = N_{ev}(t)$

A postulate Sejrsen's postulate is that

$$E = F(t)/S(t_1) \tag{15}$$

so that according to (11) and (5),

$$E = N_{ev}(t)/N(t) + N_c(t_1) + N_{ev}(t_1) + N_v(t) \tag{16}$$

Substitution of (6) and (7) into (16) yields

$$E = \frac{N_E(t) - N_{E,c}(t) - N_{E,v}(t) - N_E(t_1)}{N(t) + N_c(t) + N_T(t) + N_{E,v}(t) - N_s(t) + N_E(t_1) + N_{ev}(t)} \tag{17}$$

Using (8) (9) and (10) equation (17) becomes identical to (3) when

$$t \to t_a \tag{18}$$

$$N_{E,c}(t_1) \sim N_{E,v}(t) \sim 0 \tag{19}$$

$$N(t) \sim 0 \tag{20}$$

Comments The physical content of the assumptions required by the SIRF method can now be summarized as follows.

The proposition (11) requires that the kinetic behaviour of the molecules in the extravascular space can be deduced from the form of S(t) for $t > t_s$. For example, if the form is monoexponential it follows that the probability of translocation across the capillary wall is independent of the position of the molecules in the extravascular space and that the form of F(t) is monoexponential from t_1. In any case, a model for the kinetic behaviour of the molecules in the extravascular space must be used to prescribe the form of $F(t) = N_{ev}(t)$ at $t < t_s$.

Assumptions (18), (19) and (20) are statements about the situation at the time when S(t) has its maximum value. The first is that, at this time, all the molecules in the bolus have been either transmitted or extracted. The second is that, at the same time, none of the extracted molecules have as yet left the extravascular space. The third is that at the same time, none of the injected molecules have left the detector field

Assumption (20) can be confronted directly by experiment, but other assumptions and the proposition (11) cannot be checked by the experimental data.

It has been proposed (Paaske and Levin Nielsen 1976, Paaske 1976, Paaske 1977) that the validity of the SIRF method can be substantiated by the experimental finding that the deduced wash-out function for the transmitted molecules is identical to the observed wash-out function for a reference molecule that cannot pass the capillary membrane, *i.e.* that

$$\frac{S(t)-F(t)}{S(t_1)-F(t_1)} \quad \text{is numerically equal to} \quad \frac{S'(t)}{n} \tag{21}$$

where $S'(t)$ is the detector signal function for the reference molecules and n is the number of injected reference molecules. We show below that this observation cannot be considered as a strong proof of the validity of the SIRF method.

For any time $t > t_1$ it must be true that

$$\frac{S'(t)}{n} = \frac{N_T(t_a) - N_w(t) + N_{E,w}(t)}{N_T(t_a)} \tag{22}$$

However using (5) and (7)

$$\frac{S(t)-F(t)}{S(t_1)-F(t_1)} = \frac{N_a(t)+N_e(t)+N_T(t)+N_{E,v}(t)-N_w(t)+N_{E,w}(t)+N_{ev}(t)-F(t)}{N_a(t_1)+N_e(t_1)+N_T(t_1)+N_{E,v}(t_1)-N_w(t_1)+N_{E,w}(t_1)+N_{ev}(t_1)-F(t_1)} \tag{23}$$

The right hand sides of (22) and (23) become identical when

$$t > t_a \tag{24}$$

$$N_{E,e}(t) = N_{E,v}(t) = 0 \tag{25}$$

$$N_e(t_1) = 0 \tag{26}$$

$$F(t) = N_{ev}(t) \tag{27}$$

Thus, if the assumptions are correct, there will be numerical identity between the deduced wash-out curve for the transmitted molecules and the observed wash-out curve for the reference molecules. However, as shown by the expressions (22) and (23), an observed numerical identity does not permit the conclusion that the assumptions are correct.

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can be determined for all values of $t > t$ where t is the time at which S(t) has its maximum value The proposition is based on the following two assumptions.

(I) There exists a time $t = t > t_1$ when all the transmitted molecules have left the detector field. Thus,

$$N(t) = 0, \text{ for } t > t \quad (12)$$

It is assumed that

$$N_c(t) \sim N(t) < N_{ev}(t) \text{ for } t > t \quad (13)$$

so that, according to (5)

$$N_{ev}(t) = S(t) \text{ for } t > t \quad (14)$$

(II) The form of S(t) for $t > t_1$ can be used, by means of extrapolation back to t t_1, to determine $F(t) = N_{ev}(t)$

A postulate Sejrsen s postulate is that

$$E = F(t_1)/S(t_1) \quad (15)$$

so that according to (11) and (5),

$$E = N_{ev}(t_1)/N(t) + N_e(t_1) + N_{ev}(t_1) + N_r(t_1) \quad (16)$$

Substitution of (6) and (7) into (16) yields

$$E = \frac{N_E(t_1) - N_{E,c}(t) - N_{E,v}(t) - N_{E,a}(t_1)}{N_a(t) + N_e(t) + N_T(t) + N_{E,v}(t) - N(t_1) + N_E(t_1) + N_{ev}(t_1)} \quad (17)$$

Using (8) (9) and (10) equation (17) becomes identical to (3) when

$$t > t_a \quad (18)$$

$$N_{E,c}(t) \sim N_{E,v}(t) \sim 0 \quad (19)$$

$$N(t_1) \sim 0 \quad (20)$$

Comments The physical content of the assumptions required by the SIRF method can now be summarized as follows.

The proposition (11) requires that the kinetic behaviour of the molecules in the extravascular space can be deduced from the form of S(t) for $t > t_a$. For example, if the form is monoexponential it follows that the probability of translocation across the capillary wall is independent of the position of the molecules in the extravascular space and that the form of F(t) is monoexponential from t In any case, a model for the kinetic behaviour of the molecules in the extravascular space must be used to prescribe the form of $F(t) = N_{ev}(t)$ at $t < t_a$.

Assumptions (18) (19) and (20) are statements about the situation at the time when S(t) has its maximum value. The first is that, at this time, all the molecules in the bolus have been either transmitted or extracted. The second is that, at the same time, none of the extracted molecules have as yet left the extravascular space. The third is that at the same time, none of the injected molecules have left the detector field.

Assumption (20) can be confronted directly by experiment, but other assumptions and the proposition (11) cannot be checked by the experimental data

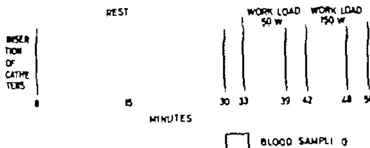


Fig. 1 Experimental design.

Material and methods

Studies were performed in 6 healthy physically well-trained volunteers with a mean age of 25.8 years (range 23–35). The investigations were carried out on an ambulatory basis. The subjects were instructed not to eat or smoke after 10 o'clock p.m. on the day prior to the investigations, which in all cases took place at 8–11 a.m.

Consent was given by each subject after complete description of the protocol. The study was approved by the Ethical Committee of the Medical Faculty at the Lund University.

Polyethylene catheters PE 160 were placed by means of the Seldinger technique in the brachial artery, the brachial vein and the femoral vein and a Gensini catheter 7 F in the left renal vein. In 3 of the subjects a Goodale-Lubin catheter 7 F was inserted into the coronary sinus through a cubital vein. The position of each catheter was controlled by X-ray. After the insertion of the catheters, the subjects rested in supine position for at least 30 min before blood samples were taken for the determination of the basal catecholamine concentrations.

Physical work consisted of bicycling in supine position for 9 min with a work load of 50 W and immediately afterwards another 9 min with a load of 150 W (Fig. 1). The bicycle used was an electrically braked variable load ergometer (Siemens-Elema). The ECG was recorded continuously during the work test.

In two of the three subjects, in which catheterization of the coronary sinus was undertaken, the maximum oxygen uptake (VO_2 max) was determined. These studies showed that a work load of 150 W represents 75 per cent of the maximum working capacity. At a work load of 150 W the mean heart rate for all participants was 163 beats/minute. Thus, it is evident that a work load of 150 W represented relatively hard work for the group as a whole.

Blood samples for the determination of catecholamines were drawn simultaneously from all catheters at rest, immediately prior to work and then during the last 3 min of work at 50 and 150 W respectively (Fig. 1).

Plasma noradrenaline and adrenaline were analyzed using a double labelling isotope derivative technique (Engelman and Portnoy 1970). In order to exclude that the blood drawn from the left renal vein was contaminated with adrenal vein blood, the concentration of cortisol was determined in the left renal vein and compared to the cortisol concentration at other levels. Cortisol was estimated using fluorometry (Clark and Rubin 1969).

When applying a work load comparable with the heaviest used in the present studies, Lange-Andersen (1968) found an increase in the coronary blood flow from about 250 ml/min at rest to about 1 000 ml/min during work. Simultaneously the kidney blood flow decreased from about 1 100 ml/min to about 300 ml/min. Using these figures and the formula,

AV-difference × plasma catecholamine concentration × plasma flow

we have calculated the net output of catecholamines from the coronary sinus and from the kidney in connection with exercise at the work load of 150 W.

Statistical analysis was performed by Student's t-test for paired observations. The values are reported as mean ± SE and the level of significance taken as $p < 0.05$.

Acta physiol scand. 1978, 104 364-369

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Plasma catecholamine levels in the coronary sinus, the left renal vein and peripheral vessels in healthy males at rest and during exercise

By

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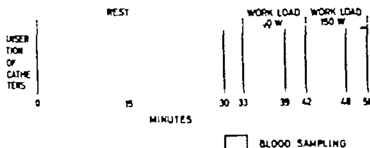
Abstract

MANHEM, P., H LECEROF and B. HÖKFELT *Plasma catecholamine levels in the coronary sinus the left renal vein and peripheral vessels in healthy males at rest and during exercise* Acta physiol. scand. 1978 *104* 364–369

Noradrenaline and adrenaline were determined in blood samples from the brachial vein, the brachial ar[illegible] the left renal vein and the femoral vein in 6 healthy males (aged 23–35 y). In 3 of the subjects catecholamines were determined also in blood from the coronary sinus. All samples were taken simultaneously in supine position after 30 min of rest and then in connection with exercise in supine position using a bicycle ergometer firstly with work load of 50 W for 9 min and secondly with a work load of 150 W for the same period of time Under resting conditions the catecholamine levels were about the same at all locations, the mean for noradrenaline being 1 59 nmol/l with a range of 1.30–2.11 nmol/l and for adrenaline 0.46 nmol/l and 0.23–0.65 nmol/l, respectively At 50 W th noradrenaline concentration increased significantly in the brachial artery the left renal vein and the femoral vein, whereas adrenaline increased significantly only in the femoral vein. At 150 W the noradrenaline content increased markedly in all samples, especially in the left renal vein (mean increase 13.02 nmol/l) and the coronary sinus (mean increase 13.06 nmol/l). Adrenaline concentration increased significantly in the brachial artery nd the femoral vein. At 150 W the mean [illegible] output of noradrenaline as estimated from the calculated flows and actual AV-differences amounted to 2.25 nmol/min from the he rt and to 0.36 nmol/min from the kidney

Key words Plasma catecholamines, renal, cardiac, ercise

In blood from healthy resting individuals the ratio of noradrenaline to adrenaline is about 5 1 but both absolute and relative amounts vary widely (Engelman and Portnoy 1970) Circulating noradrenaline is believed to originate mainly from the sympathetic nerve endings, adrenaline from the adrenal medulla. The concentration of both these catecholamines in blood increases during exercise (Kotchen *et al* 1971 Christensen and Brandsborg 1973, Chodakowska *et al* 1975 and Hansson and Hökfelt 1975) So far only few studies have been performed with the aim of defining the more precise sources of the circulating catecholamines. We have determined the catecholamine concentration in blood at different levels in the arterial and venous vascular system of healthy men at rest and after standardized light and heavy physical work.



1 Experimental design.

Material and methods

[illegible] were performed in 6 healthy physically well-trained volunteers with mean age of 25.8 years [illegible] 21-35). The investigations were carried out on an ambulatory basis. The subjects were instructed [illegible] eat or smoke after 10 clock p.m. on the day prior to the investigations, hich in all cases took [illegible] at 8-11 [illegible].

[illegible] was given by each subject after complete description of the protocol. The study was approved [illegible] Ethical Committee of the Medical Faculty at the Lund University.

[illegible] catheters PE 160 ere placed by means of the Seldinger technique in the brachial artery [illegible] brachial vein and the femoral vein and Gensini catheter 7 F in the left renal vein. In 3 of the subjects [illegible]-Lubin catheter 7 F was inserted into the coronary sinus through cubital vein. The position of [illegible] catheter was controlled by X-ray. After the insertion of the catheters, the subjects rested in supine [illegible] for at least 30 min before blood samples ere taken for the determination of the basal catechola[illegible] concentrations.

[illegible] work consisted of bicycling in supine position for 9 min ith work load of 50 W and im[illegible] afterwards another 9 min with load of 150 W (Fig. 1). The bicycle used was an electrically [illegible] variable load ergometer (Siemens-Elema). The ECG as recorded continuously during the work [illegible].

[illegible] two of the three subjects, in hich catheterization of the coronary sinus was undertaken, the maximum [illegible] uptake ($\dot{V}O_2$ max) as determined. These studies showed that ork load of 150 W represents 75 [illegible] of the maximum orking capacity. At ork load of 150 W the mean heart rat for all participants [illegible] 145 beats minute. Thus, it is evident that work load of 150 W represented relatively hard work for [illegible] group as hole.

[illegible] samples for the determination of catecholamines were drawn simultaneously from all catheters at [illegible], immediately prior to work and then during the last 3 min of work at 50 and 150 W respectively [illegible] 1).

[illegible] noradrenaline and adrenaline were analysed using double labelling isotope derivative technique [illegible] and Portacy 1970). In order to exclude that the blood draw from the left renal vein as contam[illegible] with adrenal vein blood, the concentration of cortisol as determined in the left renal vein and [illegible] to the cortisol concentration at other levels. Cortisol was estimated using fluorimetry (Clark [illegible] 1969).

[illegible] applying ork load comparable with the heaviest used in the present studies, Lange-Andersson [illegible] found an increase in the coronary blood flow from bout 250 ml/min at rest to about 1 000 ml/min [illegible] ork. Simultaneously the kidney blood flow decreased from about 1 100 ml/min to about 300 ml/[illegible]. Using these figures and the formula.

$$\text{AV-difference} - \text{plasma catecholamine concentration} \quad \text{plasma flow}$$

[illegible] have calculated the net output of catecholamines from the coronary sinus and from the kidney in [illegible] with exercise at the work load of 150 W.

[illegible] analysis as performed by Student' t-test for paired observations. The alues are reported [illegible] mean SE and the level of significance taken as p 0.05.

TABLE 1 Noradrenaline, adrenaline and cortisol in blood samples taken from the brachial artery the brachial vein, the left renal vein, the femoral vein and the coronary sinus at rest, respectively at 50 W and 150 W work load.

	Noradrenaline nmol/l						Adrenaline nmol/l						Cortisol nmol/l					
	0		50 W		150 W		0		50 W		150 W		0		50 W		150 W	
	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE	mean	± SE
A. brachialis n=6	1.33	0.22	2.15	0.41	9.54	2.39	0.63	0.45	0.74	0.13	2.17	0.60	260.0	23.6	223.0*	23.2	187.5	26.8
V. brachialis n=6	2.11	0.54	2.37	0.53	7.87	1.66	0.50	0.08	0.50	0.09	1.77	0.63	279.9	27.7	260.2	30.8	211.0*	28.8
V. renalis sin n=6	1.75	0.41	2.92	0.43	14.77	4.83	0.41	0.03	0.49	0.05	2.56	1.78	[illegible]08.0	20.6	166.6	25.4	164.5	26.2
V. femoralis n=6	1.30	0.23	2.08	0.39	9.38	2.46	0.23	0.02	0.53	0.08	1.79*	0.55	256.5	27.1	222.6	24.6	200.3	26.5
S. coronarius 3	1.44	0.16	3.06	0.32	14.5	4.65	0.49	0.06	0.67	0.09	1.86	0.39	227.6	42.5	218.3	44.8	185.0*	42.9

Denotes significant alterations compared to basal value at the level of significance where $p < 0.05$
where p 0.01 and
where p 0.001

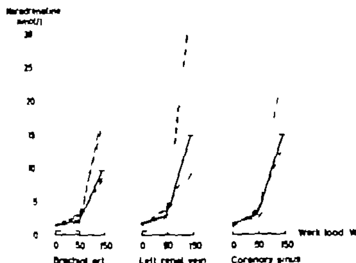


Fig. 2. Noradrenaline in blood taken from the brachial artery, the left renal vein and the coronary sinus at rest, and at 50 W and 150 W work load respectively. Broken lines indicate the values from the individual subjects, solid line indicates the mean values.

Results

The plasma cortisol concentration decreased stepwise at all sites following work and the level in the renal vein did not exceed that found in other locations at the respective times. Thus, there was no significant contamination of blood from the adrenal vein (Table I).

At rest the concentration of noradrenaline was similar in blood samples from all locations and increased uniformly at 50 W (Table I). The increase was statistically significant in samples from the brachial artery, the left renal vein and the femoral vein. At 150 W the noradrenaline content increased considerably at all sample sites. However, there was a greater increase of the noradrenaline concentration in blood from the left renal vein (n = 6) and from the coronary sinus (n = 3) as compared with other locations. Thus, concentration of noradrenaline in the left renal vein significantly exceeded the concentrations in the brachial vein, brachial artery and femoral vein (Fig.). In the coronary sinus the noradrenaline concentration at the heavier work load increased to the same level as in the left renal vein, but the increase was not statistically significant, possibly because of the relatively few subjects studied (Fig. 2).

The adrenaline concentration in samples taken at rest was of the same magnitude at all sites and increased in all locations except the brachial vein following a work load of 50 W (Table I) and at all sites following 150 W.

At 150 W the net output of noradrenaline, calculated as previously mentioned, amounted to 2.25 nmol/minute from the heart and 0.36 nmol/minute from the kidney. Similar calculations revealed no significant addition of noradrenaline from the heart and the kidney under basal conditions and at a work load of 50 W. No other significant AV-differences were registered.

TABLE I Noradrenaline adrenaline and cortisol i blood samples taken from the brachial artery the brachial vein, the left renal vein, the femoral vein and the coronary sinus at rest, respectively t 50 W and 150 W work load.

	Noradrenaline nmol/l						Adrenaline nmol/l						Cortisol nmol/l					
	0		50 W		150 W		0		50 W		150 W		0		50 W		150 W	
	mea ± SE		mean ± SE		mean ± SE		mean ± SE		mean ± SE		mean ± SE		mean ± SE		mean ± SE		mean ± SE	
A. brachialis n = 6	1.33	0.22	2.15	0.41	9.54	2.39	0.65	0.43	0.74	0.13	2.17*	0.60	260.0	23.6	223.0*	23.2	187.5	26.8
V brachialis = 6	2.11	0.54	2.37	0.53	7.87	1.66	0.50	0.08	0.50	0.09	1.77	0.63	279.9	27.7	260.2	30.8	211.0*	8.8
V renalis sin n = 6	1.75	0.41	2.92	0.45	14.77	4.83	0.41	0.03	0.49	0.05	2.56	1.78	208.0	20.6	166.6	25.4	164.5	26.2
V femoralis n = 6	1.30	0.23	2.08	0.39	9.38	2.46	0.23	0.02	0.53	0.08	1.79	0.35	256.5	27.1	222.6	24.6	200.3	6.5
S coronarius n = 3	1.44	0.16	3.06	0.32	14.5	4.65	0.49	0.06	0.67	0.09	1.86	0.39	227.6	42.5	218.3	44.8	185.0*	42.9

Denotes significant alterations compared to basal alue at the level of significance where $p < 0.05$
where p 0.01 and
where p 0.001

[illegible], K. and B. Portnoy A sensitive double-isotope derivative assay for norepinephrine and epinephrine. Circulat. Res. 1970, 26, 53–57

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Discussion

Yamaguschi (1975) stimulated the sympathetic nerves to the heart in the dog and concomitantly measured the catecholamines in the blood from the coronary sinus. He concluded that measurements of noradrenaline in blood from the coronary sinus can be used as an indication of the amount of sympathetic neurotransmittor released in heart tissue. Our results seem to support his idea.

Our findings indicate that the heart as well as the kidneys contribute to the increase in noradrenaline in blood occurring in connection with heavy work. It seems likely that the release of noradrenaline in the heart during physical work is even greater than estimated from our figures since part of the noradrenaline released is subject to re-uptake in the neuron, part is taken up by extra neuronal tissues and some is metabolized (Kopin 1977). Moreover it seems probable that some of the noradrenaline transported to the heart muscle from other locations is taken up by heart tissue (Ginn and Vane 1968).

Miura *et al* (1976) measured the concentrations of adrenaline and noradrenaline in plasma taken from different parts of the circulatory system in 22 patients with heart disease at rest and following handgrip exercise. According to their calculations the contribution of noradrenaline from the coronary sinus was rather small not only at rest but also during exercise. This difference in their results and ours probably is due to the low work load applied in the experiments of Miura *et al*.

Previously no attempts seem to have been made to calculate the noradrenaline output from the kidney during heavy work. Our results probably reflect considerably increased renal sympathetic activity at heavy work.

In healthy resting individuals Vendsalu (1960) found that the adrenaline concentration was significantly higher in the brachial artery than in the brachial vein, while the opposite was true for noradrenaline. In our study we found no such differences, either at rest or in connection with work. We can offer no definite explanation for the discrepancy between Vendsalu's findings and ours. However it may be pointed out that he investigated a larger number of individuals than we did and that the differences found by Vendsalu, although statistically significant, were rather small. Furthermore, Vendsalu used a fluorometric method to measure catecholamines, which is less capable of differentiating between noradrenaline and adrenaline as compared to the method used by us.

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I. Blood values at the end of exercise.

	Femoral venous blood			Arterio-venous difference
	pH	PCO_2 (kPa)	Lactate (mmol/l)	Lactate (mmol/l)
	6.94	10.1	20.6	−2.7
	7.00	9.3	14.1	−1.4
	7.00	8.1	20.3	−2.4

...ing exercise BD_b increased in arterial blood with the same amount as lactate pyruvate (Fig. 1). This equivalence indicates that H during this period accompanies the transport ...tate from the site of production (*i.e.* muscles working at a maximal load) to the blood ...to tissues utilizing lactate (*i.e.* liver heart and submaximally exercised muscles). During ...early part of recovery the equivalence disappeared, BD_b increased, whereas content of ...ate pyruvate did not. This finding is in agreement with previous observations that after ...imal exercise, increase in arterial BD_b is higher than corresponding accumulation of ...ate (Bouhuys *et al.* 1966, Osnes and Hermansen 1972). The present investigation is the ...t one to demonstrate that increase in hydrogen ions in blood is equivalent to the accumula-...n of lactate pyruvate during the exercise and that the discrepancy occurs first during ...recovery. It has previously been proposed that other acids than lactate (*i.e.* pyruvate ...free fatty acids) accumulate in blood and, thus, would explain the excess increase of ... (Doll *et al.* 1968). The increase of blood content of free fatty acids after exercise is, how...er, too small and the time course as described by Keul *et al.* 1969 not consistent with the ...ange in BD. In the present study it has also been shown that accumulation of pyruvate is ...o small to have any importance.

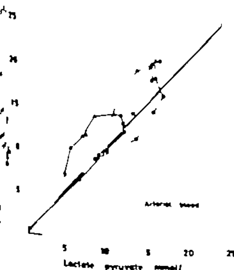


Fig. 1. Base deficit in blood (BD_b) compared with content of lactate pyruvate in blood from arteria brachialis. Samples were taken at rest ( ), during exercise (○), after 0–3 min recovery (●), and after 7 30 min recovery. Values from the same subject are joined together: —— subject K. O —— subject A R subject C. R. Content of pyruvate is at all times very small (0.5 mmol/l).

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Acid base balance in blood during exhaustive bicycle exercise and the following recovery period

By

K Sahlin A Alvestrand R. Brandt and E. Hultman

Under intense muscular exercise part of the energy is provided anaerobically by lactic acid formation The appearance of lactate in blood is associated with an increase of the hydrogen ion content It is of great importance for the understanding of acid-base balance in tissue and blood during lactic acidosis to know whether lactate is transported through the cell membrane at the same rate as the hydrogen ion or not In the present study this has been investigated by analyses of blood samples taken during exercise and the following recovery period

Methods

Three healthy male subjects aged 24–29 years participated in the experiment The nature and purpose of the study was explained to the subjects before thei[r] voluntary consent was obtained.

The [s]ubjects exercised in sitting position on [a]n [e]lectrically braked bicycle ergometer (Siemens-Elema AB, Sweden) at a continuous pedalling rate of 60 rpm. The work load calculated to lead to exhaustion after 6 min (W_{max}) was d[e]termined for each [s]ubject in a preceding session according to the method of Torntvall (1963) and was 275–300 W Subjects worked 5 min at 100 W and thereafter at W_{max} until exhaustion. Total work time was 10–10.8 min and max pulse rat[e] 170–190. After exercise the subjects rested in the supine position.

Before exercise catheters were inserted in arteria brachialis and [a] deep femoral vein. Arterial and venous blood were simultaneously collected at rest during the last 4 min of exercise and during the recovery period. Samples (5 ml) were take[n] anaerobically in heparinized syringes and were stored in ice bath and analyzed.

Acid-base data in blood (pH PCO_2, and PO_2, and Hb) were analyzed in an automatic system (Radiometer Copenhagen, Type ABL I). Base deficit [i]n blood (BD_b) was automatically calculated by the instrument from the measurements.

Immediately after the acid-base measurement, 1 ml of the blood sample was precipitated w[i]th 2 ml of 0.65 M perchloric acid. Lactate and pyruvate were analyzed by enzymatic methods on neutralized samples

Results and Discussion

Blood content of lactate and hydrogen ion increased continuously during the exercise and reached high values at the end of exercise (Table I) The release of lactate from the working muscle continued during the recovery period.

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· I. Blood values at the end of exercise.

·ct	Femoral venous blood			Arterio-venous difference
	pH	PCO_2 (kPa)	Lactate (mmol/l)	Lactate (mmol/l)
	6 94	10 1	20 6	−2 7
	7 00	9 8	14 1	−1 4
	7 00	8 1	20 3	−2.4

…ing exercise BD_b increased in arterial blood with the same amount as lactate + pyruvate …l). This equivalence indicates that H during this period accompanies the transport …tate from the site of production (i.e. muscles working at a maximal load) to the blood …to tissues utilizing lactate (i.e. liver heart and submaximally exercised muscles). During …early part of recovery the equivalence disappeared, BD_b increased, whereas content of …te pyruvate did not. This finding is in agreement with previous observations that after …imal exercise, increase in arterial BD_b is higher than corresponding accumulation of …te (Bouhuys *et al.* 1966, Osnes and Hermansen 1972). The present investigation is the …t one to demonstrate that increase in hydrogen ions in blood is equivalent to the accumula…n of lactate + pyruvate during the exercise and that the discrepancy occurs first during …recovery It has previously been proposed that other acids than lactate (i.e. pyruvate …free fatty acids) accumulate in blood and, thus, would explain the excess increase of …(Doll *et al.* 1968). The increase of blood content of free fatty acids after exercise is, how…t, too small and the time course as described by Keul *et al* 1969 not consistent with the …nge in BD In the present study it has also been shown that accumulation of pyruvate is …small to have any importance.

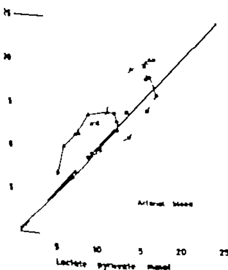


Fig. 1 Base deficit in blood (BD_b) compared with content of lactate pyruvate in blood from arteria brachialis. Samples were taken at rest (Δ), during exercise (○), after 0–2 min recovery (●), and after 7 30 min recovery A Values from the same subject are joined together —— subject K. O —— subject A. R subject C. R. Content of pyruvate is at all times very small (0 5 mmol/l).

Another possible explanation for the changed relation between BD and lactate conc is that hydrogen ions are released at a faster rate than lactate from exhausted muscle. Th hypothes is supported by a recent study of muscle metabolism (Sahlin *et al.* 1976) whe muscle samples taken during recovery from exhaustive bicycle exercise were analyzed f pH and content of lactate–pyruvate The data indicated that H^+ was transported out fr muscle in excess of lactate during the early part of recovery It has also been shown th electrically stimulated muscle in vitro has a faster efflux of H than of lactate during t recovery period (Heisler 1973)

The mechanism for hydrogen ion transport through the muscle cell membrane is n known Recent studies (Roos 1975 Mainwood and Worsley-Brown 1975) indicate th some H is transported as undissociated lactic acid Other transport mechanisms involvi movements of ions (*i.e.* H or HCO_3) ought to be influenced by the membrane potent

It has been shown (Harris *et al* 1976) that there is a rapid resynthes of creatine phospha during the first minute of recovery This resynthes is associated with a release of hydrog ions. A changed membrane permeability due to altered membrane potential, in combina tion with an intracellular hydrogen ion production related to creatine phosphate resynthesi might be the reason for hydrogen ion efflux in excess of lactate during the early part recovery

This investigation was supported by grant from the Swedish Medical Research Council (projects B73 B 1002 13A and B78 19X 2647 10A).

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Acta physiol. scand. 1978. 104 373–374

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Local lactate and exhaustion

By

PER TESCH

well established that with exhaustive exercise lactate formation will take place and
maximal levels in active muscles within a few minutes (.g. Karlsson 1971). Approxi-
ly the same lactate concentrations were obtained irrespective of performance times
sponding to 0.5–10 min. These data were obtained in homogenates of whole muscle
les and there were indications that lactate gradients existed within the biopsy sample
sson 1971). Recently microdissection technique has been developed which makes it
ble to study the lactate distribution pattern within the two main muscle f bre types,
twitch (FT) and slow twitch (ST) muscle fibres (Essén 1978). The following experiment
ndertaken in order to study the distribution of lactate in pools of different muscle
s from biopsy samples taken after short-term (1–2 min) exercise with an energy need
sponding to a level exceeding maximal oxygen uptake (approximately 120% of V_{O_2}
) 7 males characterized by a high aerobic capacity participated in the study Age, height,
ht, maximal oxygen uptake and muscle fibre type distribution in m. vastus lateralis
aged 22 (19–27) yrs, 180 (176–186) cm, 69 (63–77) kg, 66 (61 71) ml kg⁻ min and
30–62)% fast twitch (FT) fibres, respectively As observed in earlier studies a positive
tionship was present between individual V_{O_2} max and per cent ST fibres (e.g. Costill
l. 1976) (r 0.80, p 0.05). The subjects performed bicycle exercise leading to exhaustion
r 1.67 (1.05–2.10) min. Inability to maintain a certain pedal frequency (70 rev min ¹)
taken as criterion for exhaustion.

lood lactate concentration obtained 3 min after exercise corresponded to 11 7 (8.3–17.3)
ol 1⁻¹ blood.

actate concentration in freeze dried, dissected fragments of muscle fibres averaged in
fibres and ST fibres 25.8 (14 4–35 0) and 18.7 (13.6–26.5) mmol kg⁻¹ wet muscle,
pectively (p 0 05). The mean FT/ST lactate ratio was 1 4 (1 1–2.3). Lactate concentra-
n, calculated for the whole muscle (Tesch et al. 1978) averaged 20.8 (13.9–30.5) mmol
⁻¹ wet muscle and was related to the percentage of FT fibres (0.85, p 0.05 Fig. 1).
A relationship between lactate concentration and performance time was demonstrated
0 76, p 0.05, Fig. 2). In addition, it was noted that subjects with muscles rich in FT
res had higher performance capacity in the anaerobic bicycle test.

These findings indicate higher anaerobic power and capacity in subjects rich in FT f bres,
hough subjects extreme in that respect were not included in the present study Higher
ility to form lactate might be one contributing factor to anaerobic performance capacity
e results also indicated that, within one and the same muscle, FT fibres form more

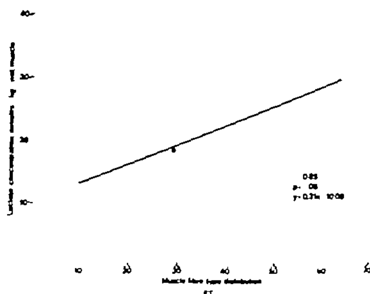


Fig 1 The relationship betw muscle fibre type distribution (% FT) and lactate concentration (n = 7).

lactate compared to ST fibres. This would also be in accordance with the different metabolic profile of the two main muscle fibre types (e.g. Sjödin 1976, Thorstensson 1976, Essén 1978) However it cannot be excluded that physical conditioning partly will contribute to the FT/ST lactate ratio observed

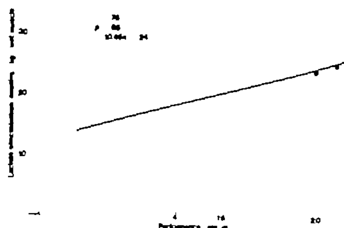


Fig. 2. The relationship between performance time (min) and lactate concentration (n = 7).

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Release of neurotensin-like immunoreactivity (NTLI) from the gut in anaesthetized dogs

By

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Neurotensin is a tridecapeptide with the sequence <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH (Carraway and Leeman 1975). It was first found in the bovine hypothalamus by Carraway and Leeman (1973). It is noteworthy that in the rat some 85% of the body's neurotensin is located in the gut and particularly in the more distal part of the small bowel (Carraway and Leeman 1976). Immunofluorescence histochemical studies have shown glandular cells in the mucosa of the ileum which react with antibodies against neurotensin (Orci *et al.* 1976, Sundler *et al.* 1977 and Helmstaedter *et al.* 1977). No plausible physiological function has been ascribed to the gut neurotensin but its cellular location suggests a hormonal role. The target tissue for neurotensin in such a hormonal role has not been found. However the peptide given by intravenous infusion in moderate doses elicits inhibition of gastric motility (Andersson *et al.* 1977), suppression of gastric acid secretion (Andersson *et al.* 1976), vasodilatation in the gastrointestinal region and a delayed vasoconstriction in subcutaneous adipose tissue (Rosell *et al.* 1976). A requirement for a hormonal action is that neurotensin is released into the blood. Here we present experimental evidence that NTLI is released from the distal ileum into the blood and into the lumen of the bowel.

The antiserum (0-7607) used in the assay of neurotensin was raised in rabbits by injection of neurotensin coupled to bovine serum albumin using 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl. The antigen was injected with Freund's complete adjuvant at 3–8 weeks intervals. This antiserum reacts with neurotensin (NT), NT(1–12), (Gln⁴)NT and (Phe)NT(1–11) but not with NT(4–13) or smaller C-terminal fragments. It shows effectively no crossreactivity with the gastric inhibitory polypeptide (GIP), the vasoactive intestinal peptide (VIP), secretin, cholecystokinin-33 cholecystokinin-39 pancreatic glucagon, substance P, bradykinin, somatostatin, gastrin-17 gastrin-34 or with trypsinized gastrin-34 which presumably contains the N-terminal heptadecapeptide. Synthetic neurotensin (Beckman) was iodinated using the chloramine T method. The assay can measure levels down to 10 pM.

To determine whether the NTLI in the blood of the nembutal anaesthetized dog could be derived from the gastro-intestinal tract, blood was collected from carotid artery, femoral vein and a mesenteric vein draining the terminal 20 cm of the ileum. The blood flow in the mesenteric vein was registered continuously by a photoelectric drop recorder to be able to measure the net outflow of NTLI from the ileum. The concentrations of NTLI were similar in plasma from the artery and the femoral vein and there was a positive V-A difference across the terminal ileum (Table I).

In these experiments a loop of the terminal 20 cm of the ileum was perfused through polyethylene tubes introduced into the lumen with a buffered solution (NaCl 70 mM, KCl

TABLE I Neurotensin-like immuno-reactivity (M ± S.E.) (Student's t-test).

Source	Concentration pM	V A pM	Release fmol min⁻¹
Carotid artery	40 ± 6.7 (8)		
Venous blood draining distal ileum	91 ± 9 7 (7)	51 ± 11	233 ± 46.8
Femoral vein	46 ± 5 9 (8)		
Perfusate distal ileum	521 ± 160 (7)		

$p < 0.01$

5 mM CaCl 25 mM Tris-HCl 70 mM pH 7.4) at a rate of 1 ml min⁻¹ The perfusate collected from the distal ileum was found to contain NTLI in all animals. The concentration in the perfusates were generally considerably higher than that in mesenteric venous plasma. The function of NTLI in the lumen is not known One possibility is that neurotensin secreted into the gut may be absorbed with or without change in passage across the intestinal wall. In 3 dogs 6 nmol neurotensin were instilled into the perfused terminal ileum and blood was sampled from the vein draining the loop. The mean concentration of NTLI rose from a baseline of 66 pM to 470 pM after 3 min It would appear then, that neurotensin in the lumen contents can be absorbed in a form which is immunoreactive.

These findings have shown that plasma from dogs contain material which reacts with an antibody directed mainly against the N-terminus of neurotensin and that such material is released into the blood by the terminal ileum. NTLI also appears in perfusates of the terminal ileum. The peptide in the lumen may represent part of a pathway for passage of neurotensin into the blood since authentic neurotensin in the fluid seems to be absorbed in an immunoreactive form.

This investigation has been supported by the Swedish Medical Research Council (3518 and 3521) and by Åk Wibergs Stiftelse We thank Dr R. Carraway (Harvard University Mass.), Dr K. Folkers (University of Texas, Austin Texas) and Dr V Mutt (Karolinska Institutet) for generous supply of various peptides.

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Acta physiol. scand. 1978. 104. 377–385

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Insulin production by pancreatic islets of obese-hyperglycemic mice cultured for one week in different glucose concentrations

By

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Abstract

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Culture of pancreatic islets isolated from obese-hyperglycemic mice (gene symbol ob) for one week in tissue culture containing widely different concentrations of glucose (3.3, 5.6 or 16.7 mM) was found to markedly influence the functional behaviour of the islet B-cells. Thus, the insulin content of islets cultured at 3.3 or 16.7 mM glucose (subphysiological or supraphysiological glucose concentrations respectively) was markedly reduced. Islets cultured in 5.6 or 16.7 mM glucose displayed normal insulin secretory response to stimulation with glucose whereas islets cultured in subnormal glucose concentration (3.3 mM) showed reduced insulin response to glucose stimulation in batch type incubations and also lacked second phase of insulin secretion in islet perifusion experiments. The rate of insulin biosynthesis of non-cultured ob/ob islets was higher than that of islets from their lean siblings but culture for one week in 3.3 mM glucose induced pronounced impairment of the insulin biosynthesis in islets of obese as well as lean mice. The present data indicate that the hyperfunction of the islets of the ob/ob mouse at least in part is a reversible phenomenon, suggesting that inherent properties of islet B-cells do not act as "primary" factors in the development of the obese hyperglycemic syndrome.

Key words: ob/ob mice, tissue culture, pancreatic islets, glucose, insulin biosynthesis, insulin release

The obese-hyperglycemic syndrome of the ob/ob mouse (gene symbol ob) is genetically determined and characterized by overeating, obesity hyperglycemia, hyperinsulinemia and insulin resistance (Ingalls *et al.* 1950 Bleisch *et al* 1952, Westman 1968). The etiology of the syndrome is unknown. Adipose tissue from lean mice transplanted to obese ob/ob mice increased markedly in size. Similarly adipose tissue of obese mice acquires the same characteristics as the host adipose tissue when grafted into lean mice (Ashwell *et al* 1976). This would argue against primary alterations of the adipose tissue as determinants of obesity in the ob/ob mice. In determining the sequential manifestation of the metabolic characteristics in young ob/ob mice, hyperinsulinemia, hypoglycemia and obesity have been found to precede the onset of hyperglycemia and insulin resistance (Dubuc 1976). Therefore, a

primary role for pancreatic islet disturbances in the development of the obese-hyperglycemic syndrome must be considered

In the present study tissue culture has been employed to evaluate the inherent properties of islets isolated from obese hyperglycemic mice and, in some experiments, from their lean litter mates. The islets were cultured for one week at different glucose concentrations, their insulin producing capacity being evaluated at the end of the culture period.

Materials and methods

Chemicals. Collagenase type CLS was obtained from Worthington Biochem Corp Freehold, New Jersey U.S.A. Tissue culture medium (TCM 199 either glucose free or containing 5.6 mM glucose), Hanks solution and calf serum was from SBL, Stockholm, Sweden. ^{125}I-insulin for the radioimmunoassay of insulin and L [4,5- ^{3}H]-leucine were from the Radiochemical Center Amersham, England. Guinea pig anti-insulin serum (AIS) was purchased from Miles Laboratories Inc., Kankakee Ill U.S.A. CNBr-activated Sepharose-4B was from Pharmacia Fine Chemicals, Uppsala Sweden and Soluene from Packard Instrument Co. Inc., Downers Grove Ill., U.S.A. The other chemicals used were all of analytical grade.

Animals and islet preparation. Non-inbred mice of both sexes with the obese hyperglycemic syndrome (genotype ob/ob Uppsala colony), 5–7 months old, weighing 50–60 g, or their lean litter mates were used. The animals were fasted over night before the experiments and they were killed by decapitation. Islets obtained by a modified collagenase method (Howell and Taylor 1968) were either used immediately in short-term experiments or transferred to plastic Petri dishes (NUNC, Roskilde, Denmark) containing tissue culture medium TCM 199 supplemented with 10% calf serum, antibiotics and the glucose concentration was adjusted to 3.3, 5.6 or 16.7 mM. For further details of the culture technique reference should be made to Andersson (1976).

Islet morphology. Cultured islets were fixed in Bouin's solution and embedded in paraffin. Sections 4 μm thick were stained with hematoxylin-eosin or aldehyde fuchsin-trichrome.

Insulin content and secretion. Groups of 10 freshly isolated or one week cultured islets were washed briefly in Hanks solution and homogenized by sonication for 30 s in 500 μl acid ethanol and extracted over night at +4°C. The extracts were stored at −20°C before the insulin assay which was performed by means of a radioimmunoassay procedure (Hedling 1972), separating free and antibody bound insulin by ethanol precipitation and using crystalline mouse insulin as standard.

Batch type incubations of one week cultured islets were performed in glass vials containing 250 μl of bicarbonate buffer (Krebs and Henseleit 1932) supplemented with 10 mM HEPES, 2 mg/ml bovine albumin and glucose and theophylline as given below. Before the experiment each vial was gassed for 1 min with O_2+CO_2 (95:5). The incubation lasted for 60 min and was performed in a water bath at +37°C with continuous shaking. At the end of the incubation the medium was removed, frozen and stored at −20°C prior to insulin assay.

Islet perifusion. The perifusion technique was adapted from Lacy *et al.* (1972) with minor modifications. Groups of 50 islets, either freshly isolated or cultured for one week, were perifused in several parallel experiments. The medium consisted of the buffer described above except for a lower albumin concentration (0.5 mg/ml). The flow rate was adjusted to 1 ml/min. Effluents were collected at 1-min or 5-min intervals and analysed for insulin content.

Insulin biosynthesis. Non-cultured or cultured islets in groups of 10 to 20 were incubated for 120 min in the presence of 100 μCi/ml L [4,5- ^{3}H]-leucine (spec. act. 58 Ci/mmol). Labelled proinsulin and insulin in the homogenized islets were separated from other islet proteins by means of an immune binding technique described in detail by Berne (1975). One aliquot of the islet homogenate was taken for DNA measurements as described by Kissane and Robins (1958).

Results

Islet morphology. It was found that the large islets with a diameter exceeding approximately 250 μm, which can be obtained from the ob/ob mouse had a tendency to develop central necrosis, as evidenced by a dense white spot appearing in the ce[illegible] of the explant when

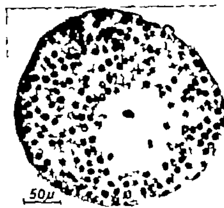


Fig. 1 A Bouin-fixed and hematoxylin-eosin stained section of an ob/ob islet cultured for one week at 16.7 mM glucose. There ... central area of necrotic cells is surrounded by normal epithelioid islet cells.

...ved in the stereo microscope during the culture. Such areas were most frequently observed in islets cultured in 16.7 mM glucose and were very seldom seen in cultured islets isolated from the lean control mice. Fig. 1 shows a light microscopical section of a cultured ob/ob mouse islet with a central area of necrotic cells.

Insulin content. As can be seen in Fig. 2 all groups of cultured islets displayed a lower insulin content than the non-cultured ones. The highest value for the cultured islets was obtained for those cultured in 5.6 mM glucose (p 0.02 vs. islets cultured in 16.7 mM glucose, p 0.005 vs. islets cultured in 3.3 mM glucose), which contained about half as much insulin as that found in the freshly isolated islets (p 0.01). The high (16.7 mM) and the low glucose (3.3 mM) cultured islets had an insulin content, which was reduced to about one third of that in the non-cultured islets (p 0.001).

Insulin secretion in batch experiments. As can be seen in Fig. 3 the ob/ob islets cultured in 3.3 mM glucose were found to respond to an acute glucose load with a significantly (p 0.05) increased insulin secretion only at the highest glucose concentration tested (27.8 mM). On the other hand, islets cultured for one week in 5.6 mM glucose displayed an increased rate of insulin secretion already at a concentration of 8.4 mM glucose reaching a maximum at 16.7 mM glucose. In a separate series of experiments with the islets cultured in 5.6 mM glucose, theophylline was excluded from the incubation medium used in the short-term expts. Also in this case glucose exerted a stimulatory effect on the insulin release, the maximal secretory rate being almost 10 times higher at 27.8 mM glucose as compared to the basal rate in the total absence of glucose.

The islets cultured in 16.7 mM glucose for one week had a basal secretory rate per islet, which was about four times higher than those recorded for the other two groups of cultured islets (p 0.001). Moreover at all glucose concentrations these high-glucose cultured islets secreted most insulin, although the difference from the islets cultured in 5.6 mM glucose did not reach statistical significance at the two highest concentrations.

Insulin secretion in islet perifusion experiments. The release of insulin from cultured islets obtained from obese-hyperglycemic mice during perifusion with 2.8 and 28 mM glucose is shown in Fig. 4. The rate of insulin output at the low glucose concentration was shown to be dependent on the glucose environment during the culture period, the highest prestimulatory

primary role for pancreatic islet disturbances in the development of the obese-hyperglycemic syndrome must be considered

In the present study tissue culture has been employed to evaluate the inherent properties of islets isolated from obese-hyperglycemic mice and, in some experiments, from their lean litter mates. The islets were cultured for one week at different glucose concentrations, their insulin producing capacity being evaluated at the end of the culture period.

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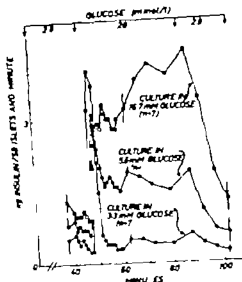


Fig. 4. Fifty ob ob islets cultured for one week at 3 different glucose concentrations were perfused with 3 or 21 mM glucose during the intervals given by arrows.

Insulin biosynthesis. The rates of proinsulin + insulin biosynthesis in non-cultured and cultured islets are shown in Table I. At 3.3 mM glucose, freshly isolated, non-cultured islets obtained from obese mice synthesized significantly more proinsulin + insulin than islets of lean mice. The percentage of total islet radioactivity incorporated into the proinsulin + insulin fraction was also significantly greater in islets of obese animals.

A high glucose concentration (16.7 mM) increased the incorporation into proinsulin + insulin in non-cultured islets from lean as well as obese mice, although the statistical significance was poor for the latter islets (p 0.10). Also at this glucose concentration the islets of the obese mice synthesized more proinsulin + insulin than islets of lean mice. As evidenced by the increased percentage, glucose preferentially stimulated incorporation into proinsulin + insulin as compared to other islet proteins in both sets of islets. These increases, however, achieved statistical significance only with islets of lean animals.

Culture for one week at a low glucose concentration (3.3 mM) drastically reduced the biosynthetic activity of islets from both groups of animals. The islets of obese mice, however, still synthesized more proinsulin + insulin than did islets of lean animals during the -hour incubation period at 16.7 mM glucose. The biosynthetic rate at 3.3 mM glucose was very low and difficult to differentiate from blank values.

Exposure to 16.7 mM glucose for one week partially eliminated the difference between islets from lean and obese mice, in that the biosynthesis of proinsulin + insulin was similar at low glucose concentrations. These rates were of the same order of magnitude as in non-cultured islets. The percentage of total islet label being incorporated into proinsulin + insulin was, however, still greater in islets of obese mice. A high glucose concentration in the short-term experiments failed to enhance the proinsulin + insulin synthesis in islets of lean mice cultured in high glucose environment. Under the same conditions, islets of obese mice maintained their insulin biosynthetic response to high glucose in absolute terms.

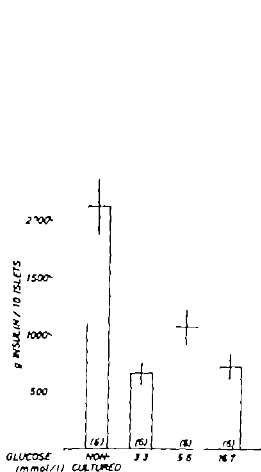


Fig. 2

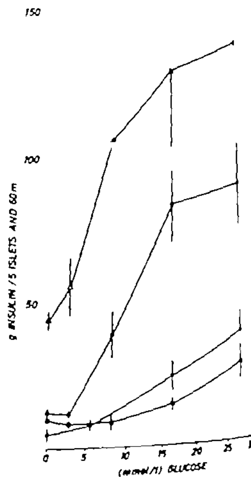


Fig. 3

Fig. 2. Mean insulin content (±S.E.) of islets isolated from ob/ob mice. The islets were either fresh isolated or cultured for one week at 3 different glucose concentrations.

Fig. 3. Insulin release from one week cultured ob/ob islets as estimated in batch-type expts, the glucose concentration of the short term incubation media being given at the abscissa. The islets had been cultured either in 3 3 mM glucose (open quadrangles), 5 6 mM glucose (open circles) or 16.7 mM glucose (open triangles). All short term incubations were performed in the presence of 5 mM theophylline except for those denoted by filled circles. The values are given as means ± S.E. of 4 expts.

release being observed with islets cultured at 16 7 mM glucose ($p < 0.005$ vs culture in 5.6 mM glucose, $p < 0.001$ versus culture in 3 3 mM glucose). This is in agreement with the batch-type expts. (*cf* Fig. 3)

Upon raising the glucose concentration of the perifusate to 28 mM the islets responded with a pronounced first phase of insulin release. The magnitude and the temporal characteristics of this early insulin response seemed to be independent of the glucose concentration of the culture medium. On the other hand, the glucose milieu during the culture period was a decisive determinant of the second phase of insulin response to high glucose stimulation. Thus, after culture at 3.3 mM glucose there was a little or no sustained insulin response to 28 mM glucose. Islets cultured at 5 6 as well as 16 7 mM glucose displayed a typical second phase of insulin output, the rate of release per islet being most pronounced after culture at 16 7 mM glucose.

content during such a prolonged exposure to high glucose to less than 10% (Andersson *et al.* 1974).

The most conspicuous finding in the present investigation was, however the ability of islets isolated from obese mice to adapt to changes in local environment in a similar way as the adaptation of islets of normal mice to alterations in tissue culture conditions, as previously extensively investigated in our laboratory (Andersson *et al.* 1974 Andersson *et al.* 1976). Thus, after one week in tissue culture, differences in proinsulin + insulin biosynthesis between obese and lean mice were greatly reduced. In addition, the insulin secretory response to glucose after tissue culture was highly dependent on the glucose concentration of the culture medium. In islets of obese mice, characteristics such as absolute magnitude of insulin response, dose-response relationships and time-dependent profile of hormone secretion were all modified by ambient glucose concentrations during the culture period. These islets, deprived of the anatomical and metabolic environment that constitutes the obese-hyperglycemic syndrome, thus rapidly adapt to new external conditions. This conclusion does not, however imply that the pancreatic islets have no central role to play in the pathogenesis of the obese-hyperglycemic syndrome (*cf* Strautz 1970 Beloff-Chain *et al* 1973 Lavine *et al* 1977). The hyperinsulinemia in the face of hypoglycemia observed early in the development of the syndrome (Dubuc 1976) suggests that factors other than blood glucose are major determinants of B-cell function in young ob/ob mice. Strautz (1970) found that implantation of islets isolated from lean mice into obese mice ameliorates the obese-hyperglycemic syndrome. Parallel observations have been made in New Zealand obese mice (Gates *et al* 1972). These findings could indicate that normal islets do not react to the metabolic environment in the same way as the islets of developing ob/ob mice. In disagreement with our present findings, this would suggest that there is an intrinsic abnormality in the B-cells of ob/ob mice.

An alternative explanation is that the normal location of the islets is of primary importance for their functional integrity in normal as well as obese mice This suggestion agrees with the hypothesis of hypothalamic-islet axis that may have an altered functional "set-point" in at least some forms of obesity (Nagamaski 1972, Asplund 1977). B-cells are clearly under the control of the autonomic nervous system (for review see Woods and Porte 1974), and hypothalamic manipulation causes not only obesity but also acute (Curry and Joy 1974, Rohner *et al* 1977) as well as long-term (Hustvedt and Løvø 1972) effects on insulin secretion. Recent observations by Malaisse Lagae *et al.* (1977) in C57B1/6J-ob mice, and by Gates and Lazarus (1977) in NZO mice suggest that the hypothalamic abnormality in turn might be due to failure of a feedback signal from the islets, mediated by pancreatic polypeptide If these results are confirmed, they would explain the observations of Strautz (1970) and Gates *et al* (1972) cited above, as well as being totally compatible with our own results, which suggest normal B-cell adaptation in ob/ob mice when studied devoid of their hypothalamic connexion. Our results argue strongly against inherent properties of the B-cells as "primary" factors responsible for the obese-hyperglycemic syndrome.

These studies were supported by grants from the Swedish Medical Research Council, the Swedish Diabetes Association and the Nordic Insulin Foundation. We wish to thank Mrs Margareta Engkvist and Miss Astrid Nordin for their skilful technical assistance and Mrs Ingar Eriksson for typing this manuscript.

TABLE I Insulin biosynthesis of non-cultured and cultured islets. Islets were isolated either from ob(mice or from their lean litter mates. Insulin biosynthesis of freshly isolated islets or islets cultur for one week in 3.3 or 16.7 mM glucose was estimated by measuring the incorporation tritiated leucine at 3.3 or 16.7 mM glucose for 2 h into both the proinsulin + insulin fractio (PI–I) and TCA precipitable protein fraction of the islet homogenate. The values in column refer to the percentage of the total incorporated radioactivity represented by the PI-I fractio as calculated from each individual observation. DNA was estimated by means of a fluoropho metric method. Means ± S.E. are given as well as the statistical significance of the differe between lean and obese mice ($= p < 0.05$ $= p < 0.001$ $= p < 0.001$) or between short-ter incubation at 3.3 or 16.7 mM glucose ($^a = p < 0.01$ $^b = p < 0.001$). Numbers of observations a gi en within parenthesis.

Incubation (mM glucose)	3.3 PI I (cpm × 10⁻³/μg DNA .h)			16.7 PI-I (cpm 10⁻³/μg DNA 2h)	
Islet specimen					
Lean non-cultured	23 3 ± 4.2	19 4 ± 1 4	(9)	57.3 ± 7 1^b	26.3 ± 1 4^a (
Obese non-cultured	59 7 ± 13 6	25 0 ± 2.0	(11)	89.5 ± 11.6	32.3 ± 3 0 (
Lean cultured in 3.3 mM glucose	—	—		6.8 ± 2.1	2.6 ± 0 5 (
Obese cultured in 3.3 mM glucose	—	—		13.1 ± 2.0*	4 4 ± 0.5* (
Lean cultured in 16.7 mM glucose	39 7 ± 7 6	9.2 ± 0.9	(9)	7.3 ± 5.8	9 4 ± 1.5 (
Obese cultured in 16.7 mM glucose	45 6 ± 8.2	15.5 ± 0 8	(7)	73.9 ± 18.7*	17.2 ± 1 5** (

Discussion

The tissue culture procedure used in the present series of experiments has been thoroug evaluated previously and it has proven a useful investigative tool in the study of prolong effects of various substances on the structure and function of pancreatic islets (Anders and Hellerström 1976) Thus, by the use of islets isolated from normal mice valuable inf mation on the regulatory mechanisms of insulin production has accumulated (Anders 1974 Andersson *et al* 1974) In the present study the tissue culture technique has been u to characterize the possible inherent properties of islets of obese-hyperglycemic m Certain aspects of insulin production by cultured islets of obese-hyperglycemic mice h previously been published by other authors (Buitrago *et al* 1975).

Previously islet hyperplasia (Cameron *et al* 1972), lower B-cell secretory gran content (Björkman *et al* 1963) and greater sensitivity of the insulin releasing mechanism low glucose concentrations (Lavine *et al* 1977) have been documented in this strain. T present study adds some further evidence for a modified B-cell function in obese mice wh compared to lean mice. Thus, freshly isolated islets of obese animals incorporated m radioactivity into proinsulin + insulin than islets of lean mice at low as well as high gluc concentrations. This was partly due to a larger proportion of the total islet biosynthe activity being diverted to proinsulin + insulin production in obese animals. Furthermo the insulin content of the islets of the obese mice was reduced to about one third after cult for one week in 16.7 mM glucose, whereas normal mouse their insu

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Table I. Gastrin release (pg/min) into the antral lumen during a) perfusion with 0.15 M NaCl pH 6.8, 0.15 M NaCl pH 3–4, and 0.1 M HCl and b) 0.15 M NaCl pH 6–8, 0.1 M citrate buffer pH 5, 0.1 M citrate buffer pH 3 and 0.1 M HCl. Note that the gastrin release remains relatively unchanged when pH is lowered from 6.8–1.2.

	NaCl pH 6 8	NaCl pH 4	HCl pH 1	
	600	500	550	
	1 200	1 300	1 375	
	NaCl pH 6 8	Citrate buffer pH 5	Citrat buffer pH 3	0 1 M HCl
	800	700	750	600
	400		500	400

Methods

Cats were anesthetized with chloralose and urethane (50 and 100 mg/kg). Their antra were isolated by placing ligatures at the antrum-corpus border and at the pylorus. Antral perfusions were performed with warm solutions of various pHs via a pyloric catheter. The perfusate left the antrum against a slight pressure (10 cm H_2O) via a small cannula inserted into the antral wall. The rate of the perfusion was 1–2 ml/min. The pH of the outflowing perfusates were recorded.

The following solutions were used: 0.15 M NaCl (pH 6.8 and 4), 0.1 M HCl, 0.15 M $NaHCO_3$ (pH 8), 0.1 M phosphate buffer pH 7.4, 0.15 M Tris buffer (pH 8), 0.1 M and 0.01 M NaOH (pH 13 and 12), and 0.1 M citrate buffer (pH 3 and 5). The perfusion time varied between 30 and 100 min. After having passed the antrum, the perfusate was collected in tubes kept on ice. Samples were taken every 10 min and pH was measured and adjusted to 7. After being boiled for 10 min the samples were frozen until assayed for gastrin. The gastrin output into the antral lumen was then calculated from the gastrin concentration of the perfusates and the rate of the perfusion (ml/min).

During the experiments repeated blood samples for gastrin determinations were taken from the femoral artery. Gastrin was determined by radioimmunoassay as described by Nilsson (1975), using antiserum 2604 kindly supplied by professor Jens Rehfeld, University of Aarhus, Denmark.

Results

1. Perfusion with 0.15 M NaCl pH 6.8, 0.15 M NaCl pH 4, 0.1 M citrate buffer pH 3 and 5 and 0.1 M HCl

In these experiments the antrum was successively perfused with solutions of falling pH for 30 min periods. The gastrin release during each perfusion period was calculated based on the values from 3 consecutive 10 min samples. In all experiments the perfusion was started with 0.15 M NaCl pH 6.8 and was finished with 0.1 M HCl. In between the effect of either 0.15 M NaCl pH 4 (n = 2) or citrate buffer pH 3 and 5 (n = 2) was tested. As demonstrated in Table I variations of the antral acidity within the pH range 1.2–6.8 was not accompanied by any significant changes in the basal intraluminal gastrin output. Especially notable is the fact that acidification of the antral perfusates from 6.8 down to 1.2 did not result in any significant inhibition of the basal gastrin release.

2. Perfusion with 0.1 M HCl and 0.15 M NaCl pH 4

The antrum was initially perfused with 0.1 M HCl (pH 1.2) and then with 0.15 M NaCl (pH 6.8) for 40 min each. The gastrin release per minute into the antral lumen based on 4 consecutive 10 min samples during perfusion, with the different solutions is seen in Table II a and b.

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Effect of intraantral pH on basal gastrin release into the circulation and antral lumen in anesthetized cats

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Abstract

UVNÄS-WALLENSTEN K *Effect of intraantral pH on basal gastrin release into the circula[illegible] and antral lumen in anesthetized cats* Acta physiol scand. 1978 *104* 386–393.

In acute experime ts on cat antral pouches were perfused with solutions of different pH (1 13) Aft[illegible] antrum passage the gastrin levels n the perfusates were measured with radioimmunoassay and th[illegible] amou ts of gastrin released nto the antral lumen per minute calculated. The venous gastrin levels we[illegible] determined concomitantly Small mount of gastrin (1 000–1 500 pg/min) were released into the antr[illegible] during perfusion with 0 1 M HCl Subsequent perfusion with 0.15 M NaCl (pH 6.8) did not significant[illegible] increase the release of gastrin. On the othe hand, 0.1 M phosphate buffer (pH 7 4) caused a dramat[illegible] ugmentation of the gastrin output i to the antral lumen (~ 17 fold). A concomitant increase of peripher[illegible] gastrin levels was observed. Also other alkaline solutions such as 0.15 M $NaHCO_3$ (pH 8), 0 15 M Tri[illegible] buffer (pH 8) r 0 01 and 0 1 M NaOH (pH 12 and 13) promoted the release of gastrin. It is discuss[illegible] whether the gastrin release at lkaline pH is nduced by the lkaline pH itself or by anions such as HPO_4 HCO and OH The apparent ffect of pH could then be due to the formation of these ions at higher pH

The inhibitory effect of antral acidification on the gastric acid secretion was first describe[illegible] by Dragstedt *et al* (1951) and by Woodward *et al* (1954 and 1957). They ascribed th[illegible] reduction of the HCl-secretion to a decreased release of gastrin into the circulation an[illegible] proposed the existence of an antral pH control of the gastrin release mechanism. Mo[illegible] conclusions, including those mentioned above, concerning the effect of antral pH o[illegible] gastrin release were based on indirect evidence, the acid secretory responses. Toda[illegible] however it has been directly shown with radioimmunoassay that acidification of th[illegible] antrum reduces the gastrin release into the circulation caused by different kinds of stimul[illegible] tion, such as sham feeding (Nilsson *et al* 1972) antral perfusion with ACh (Thompson *et a*[illegible] 1970) or with peptone (Konturek *et al* 1974) etc. However the reports concerning th[illegible] effects of intraantral pH on the basal gastrin release are less consistent The aim of th[illegible] present investigation was therefore to study the influence of intraantral pH on this type [illegible] gastrin release. Cats were provided with acute antral pouches, which were then perfuse[illegible] with solutions of various pH Since gastrin is released both into the antral lumen and int[illegible] the circulation (Uvnäs-Wallensten and Rehfeld 1976 Uvnäs-Wallensten 1977) antr[illegible] perfusates as well as blood samples were analyzed for their gast[illegible] nt.

Table I Gastrin release (pg/min) into the antral lumen during a) perfusion with 0.15 M NaCl pH 6 8, 0.15 M NaCl pH 3–4, and 0 1 M HCl and b) 0.15 M NaCl pH 6–8, 0.1 M citrate buffer pH 5, 0.1 M citrate buffer pH 3 and 0.1 M HCl. Note that the gastrin release remains relatively unchanged when pH is lowered from 6 8–1.2.

	NaCl pH 6 8	NaCl pH 4	HCl pH 1	
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Methods

Cats were anesthetized with chloralose and urethane (50 and 100 mg/kg). Their antra were isolated by applying ligatures at the antrum-corpus border and at the pylorus. Antral perfusions were performed with warm solutions of various pH via a pyloric catheter. The perfusate left the antrum against a slight pressure (10 cm H_2O) via a small cannula inserted into the antral wall. The rate of the perfusion was 2 ml/min. The pH of the outflowing perfusates were recorded.

The following solutions were used. 0.15 M NaCl (pH 6 8 and 4), 0.1 M HCl, 0 15 M $NaHCO_3$ (pH 8), 0.1 M phosphate buffer pH 7 4, 0 15 M Tris buffer (pH 8), 0.1 M and 0 01 M NaOH (pH 13 and 12), and 0.1 M citrate buffer (pH 3 and 5). The perfusion time varied between 30 and 100 min. After having passed the antrum, the perfusate was collected in tubes kept on ice. Samples were taken every 10 min and pH was determined and adjusted to 7. After being boiled for 10 min the samples were frozen until assayed for gastrin. The gastrin output into the antral lumen was then calculated from the gastrin concentration of the perfusate and the rate of the perfusion (ml/min).

During the experiments repeated blood samples for gastrin determinations were taken from the femoral artery. Gastrin was determined by radioimmunoassay as described by Nilsson (1975), using antiserum 2604 kindly supplied by professor Jens Rehfeld, University of Aarhus, Denmark.

Results

1. Perfusion with 0 15 M NaCl pH 6.8, 0.15 M NaCl pH 4, 0 1 M citrate buffer pH 3 and 5 and 0 1 M HCl

In these experiments the antrum was successively perfused with solutions of falling pH for 30 min periods. The gastrin release during each perfusion period was calculated based on the values from 3 consecutive 10 min samples. In all experiments the perfusion was started with 0.15 M NaCl pH 6.8 and was finished with 0 1 M HCl. In between the effect of either 0.15 M NaCl pH 4 (n = 2) or citrate buffer pH 3 and 5 (n = 2) was tested. As demonstrated in Table I variations of the antral acidity within the pH range 1.2–6.8 was not accompanied by any significant changes in the basal intraluminal gastrin output. Especially notable is the fact that acidification of the antral perfusates from 6.8 down to 1.2 did not result in any significant inhibition of the basal gastrin release.

2. Perfusion with 0.1 M HCl and 0.15 M NaCl pH 4

The antrum was initially perfused with 0.1 M HCl (pH 1.2) and then with 0.15 M NaCl (pH 6 8) for 40 min each. The gastrin release per minute into the antral lumen based on 4 consecutive 10 min samples during perfusion, with the different solutions is seen in Table II a and b

TABLE II Gastrin release (pg/min) into the antral lumen during antral perfusion with 0.1 M HCl (a) or 0.1 M NaCl (b). The gastrin release was determined during 4 consecutive 10 min periods at each pH and amounted to ~1 300 pg/min (350–2 125) and ~1 400 pg/min (290–2 500) respectively

TABLE II a. Antral perfusion with 0.1 M HCl.

Cat no	0–10 min	10–20 min	20–30 min	30–40 min	
1	2 000	2 500	2 000	2 000	2 125
2	400	500	500	400	450
3	500	300	300	300	350
4	2 000	2 500	2 000	2 000	2 125
				M	1 262.5

TABLE II b. Antral perfusion with 0.15 M NaCl.

Cat no	40–50 min	50–60 min	60–70 min	70–80 min	
1	2 550	2 400	2 000	2 400	2 337
2	600	500	500	400	500
3	300	200	300	350	290
4	3 000	2 500	2 500	2 000	2 500
				M	1 406

On the average 1 300 pg/min (350–2 125) were released into the antral lumen during perfusion with HCl and 1 400 pg/min (290–2 500) during perfusion with NaCl. Obviously a shift from a strongly acid perfusion solution (0.1 M HCl) to an almost neutral one (0.15 M NaCl) did not increase the intraluminal gastrin secretion. The peripheral gastrin levels also remained unchanged.

3 *Perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4 (n = 8)*

In this series of experiments, the antrum was consecutively perfused with 0.1 M HCl (pH 1.2) and 0.1 M phosphate buffer (pH 7.4) for 40 min each. The results which are presented in Table III a and b show that ~1 500 pg/min (150–2 625) of gastrin were released into the antral lumen during perfusion with acid, whereas the release during perfusion with phosphate buffer amounted to ~24 000 pg/min (3 675–50 500). The ratio between the gastrin release observed during perfusion with HCl and phosphate buffer was calculated in each cat (Table IV). On the average the release rate increased 17 fold (6–34) when HCl was changed to phosphate buffer pH 7.4. A simultaneous increase of peripheral gastrin levels was observed (Fig. 1).

On perfusion with phosphate buffer gastrin release remained increased for 30–100 min. However if acid was introduced into the antrum while the gastrin release was still elevated, an immediate return towards prestimulatory gastrin levels occurred (Fig. 1) (n = 5). The increased gastrin release caused by antral perfusion with phosphate buffer was independent on intact vagal innervation since phosphate buffer also caused a release of gastrin in cats subjected to previous truncal vagotomy (n = 2). The pH of the phosphate buffer was adjusted to 3 and 5 by addition of 0.1 M HCl (n = 2). No gastrin release was induced by phosphate buffers of pH 3 and 5. 0.1 M phosphate pH 7.4 was diluted to 1 ... 1:100 and 1:1 000 by

TABLE III Gastrin release (pg/min) into the antral lumen during antral perfusion with 0.1 M HCl (a) and 0.1 M phosphate buffer pH 7.4 (b). The gastrin release was determined during four consecutive 10 min periods at each pH and amounted to ~1 500 pg/min (150–2 625) and 24 000 (3 675–50 500) respectively

III a Gastrin release into the antral lumen (pg/min)

Cat no.	0–10 min	10–20 min	20–30 min	30–40 min	Mean
I	2 800	3 000	3 000	2 500	2 625
II	700	800	900	1 000	850
III	100	200	100	200	150
IV	2 900	2 000	2 000	1 500	2 000
V	2 000	1 500	1 000	1 000	1 375
VI	3 000	2 500	2 000	1 500	2 250
VII	1 000	2 000	1 000	1 500	1 375
VIII	2 000	1 000	900	900	1 200
					1 500

TABLE III b Gastrin release into the antral lumen (pg/min)

Cat no.	40–50 min	50–60 min	60–70 min	70–80 min	Mean
I	40 000	40 000	80 000	42 000	50 500
II	16 000	15 000	9 000	2 000	10 500
III	2 200	4 500	4 400	3 800	3 675
IV	8 000	20 000	17 500	22 500	17 000
V	11 000	12 000	7 500	4 000	8 625
VI	32 500	60 000	32 500	20 000	41 000
VII	17 500	22 500	28 000	15 000	20 750
VIII	30 000	30 000	50 000	50 000	40 000
					24 000

addition of 0.15 M NaCl. The 0.01 and 0.001 M phosphate buffer caused a significant release of gastrin, (10-fold and 2-fold increase respectively) whereas the 0.0001 M solution failed to do so (n = 2).

Perfusion with HCl–Tris buffer pH 8, n = 3 or $NaHCO_3$, pH 8, n = 3

Both these buffers caused an increase of the gastrin output into the lumen and into the circulation. A representative experiment is illustrated in Fig. 2.

TABLE IV The ratio between the gastrin release into the antral lumen during perfusion with 0.1 M HCl and with 0.1 M phosphate buffer pH 7.4

Cat no.	Ratio between gastrin release rate during perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4
I	1:12
II	1:12
III	1:25
IV	1:9
V	1:6
VI	1:18
VII	1:15
VIII	1:33
Mean	1:17

TABLE II Gastrin release (pg/min) into the antral lumen during antral perfusion with 0.1 M HCl (a) a 0.1 M NaCl (b). The gastrin release was determined during 4 consecutive 10 min periods at e pH and amounted to ~1 300 pg/min (350–2 125) and ~1 400 pg/min (290–2 500) respectivel

TABLE II a. Antral perfusion with 0.1 M HCl.

Cat no.	0–10 min	10–20 min	20–30 min	30–40 min	
1	2 000	2 500	2 000	2 000	2 125
2	400	500	500	400	450
3	500	300	300	300	350
4	2 000	2 500	2 000	2 000	2 125
				M	1 262.5

TABLE II b. Antral perfusion with 0.15 M NaCl.

Cat no.	40–50 min	50–60 min	60–70 min	70–80 min	
1	2 550	400	2 000	2 400	337
2	600	500	500	400	500
3	300	200	300	350	290
4	3 000	2 500	2 500	2 000	2 500
				M	1 406

On the average 1 300 pg/min (350–2 125) were released into the antral lumen dur perfusion with HCl and 1 400 pg/min (290–2 500) during perfusion with NaCl. Obvious a shift from a strongly acid perfusion solution (0.1 M HCl) to an almost neutral one (0.1 M NaCl) did *not* increase the intraluminal gastrin secretion. The peripheral gastrin leve also remained unchanged

3 *Perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4 (n = 8)*

In this series of experiments, the antrum was consecutively perfused with 0.1 M HC (pH 1.2) and 0.1 M phosphate buffer (pH 7.4) for 40 min each. The results which ar presented in Table III a and b show that ~1 500 pg/min (150–2 625) of gastrin were release into the antral lumen during perfusion with acid whereas the release during perfusion wit phosphate buffer amounted to ~24 000 pg/min (3 675–50 500). The ratio between the gastrin release observed during perfusion with HCl and phosphate buffer was calculated fr each cat (Table IV) On the average the release rate increased 17-fold (6–34) when HCl was changed to phosphate buffer pH 7.4 A simultaneous increase of peripheral gastrin levels was observed (Fig. 1).

On perfusion with phosphate buffer gastrin release remained increased for 30–100 min. However if acid was introduced into the antrum while the gastrin release was still elevated, an immediate return towards prestimulatory gastrin levels occurred (Fig. 1) (n = 5). The increased gastrin release caused by antral perfusion with phosphate buffer was independent on intact vagal innervation since phosphate buffer also caused a release of gastrin in cats subjected to previous truncal vagotomy (n = 2) The pH of the phosphate buffer was adjusted to 3 and 5 by addition of 0.1 M HCl (n = 2) No gastrin release was induced by phosphate buffers of pH 3 and 5 0.1 M phosphate pH 7.4 was diluted t 1100 and 1 000 by

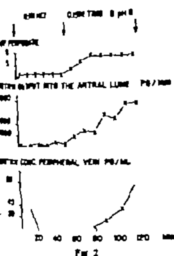


Fig 2

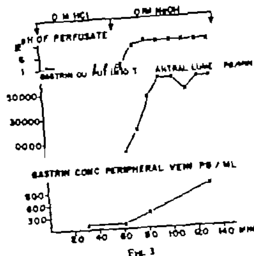


Fig 3

Fig 2 Gastrin release into the antral lumen (pg/min) and peripheral gastrin levels during perfusion with 0.1 M HCl (60 min) and 0.15 M Tris buffer pH 8 (70 min). Note the parallel increase of intraluminal gastrin release and peripheral gastrin levels in response to antral perfusion with Tris buffer

Fig 3 Gastrin release into the antral lumen (pg/min), and peripheral gastrin levels (pg/ml) during perfusion with 0.1 M HCl (60 min) and 0.1 M NaOH (80 min). Both the intraluminal output of gastrin and the peripheral gastrin levels increased dramatically in response to perfusion with NaOH

acid secretion increased manyfold in dogs with transplanted antra. Addition of acid to the transplanted antra inhibited the gastric acid secretion. The basal release of gastrin was assumed to be controlled by the antral pH, the release being depressed by acidification. It should be stressed that gastrin measurements could not be performed at that time. Furthermore, the antra transplanted to the duodenum and the colon might have been exposed to gastrin releasing agents present in the intestinal contents not present in an empty antrum in situ.

Our results show that in the anesthetized cat basal gastrin release is not appreciably affected by changes of pH in the range of 1 7 They agree with those of McLaughlin *et al.* (1975) who found no difference in the output of gastrin into the portal vein of cats during antral perfusion with 0.15 M NaCl at pH and 8. In contrast to basal gastrin release stimulated gastrin release is efficiently inhibited by antral acidification.

Thus in the cat during antral perfusion with HCl the gastrin release responses induced by electrical vagal stimulation were only 30% of those induced by an identical stimulation during antral perfusion with 0.15 M NaCl (Uvnäs-Wallensten *et al.* 1977)

By which mechanisms does antral perfusion with phosphate buffer and solutions of bicarbonate and NaOH stimulate the release of gastrin? The gastrin release caused by phosphate buffer pH 7.4 was sometimes enormous (30-fold increase) indicating that phosphate ions might be specifically involved in the release activation. To study this possibility phosphate buffers of lower pH were administered into the antrum. No gastrin release was induced by phosphate buffers of pH 3 or 5 whereas large amounts were released when their pH was increased to 7 Below pH 6 H_2PO is the dominating anion whereas HPO predominates around pH 7 indicating that HPO ions may be the gastrin releasing

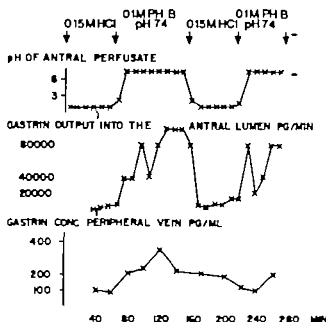


Fig. 1 Gastrin release into the antral lumen (pg/min), and peripheral gastrin levels (pg/ml) during antral perfusion with 0.1 M HCl and 0.1 M phosphate buffer pH 7.4 during two consecutive min periods. Intraluminal gastrin release as well as peripheral gastrin levels increased during perfusion with phosphate buffer. In this experiment the intraluminal release of gastrin lasted as long as the perfusions with phosphate buffer were performed, but decreased as soon as HCl was introduced into the antral pouch.

5 Perfusion with 0.1 M HCl 0.01 or 0.1 M NaOH n = 3

0.01 M NaOH (pH 12) caused only a slight (two-fold) increase of the gastrin release into the peripheral blood and into the lumen, whereas 0.1 M NaOH (pH 13) released large amounts of gastrin both into the lumen (20-fold increase) and into the circulation. A typical experiment is represented in Fig. 3

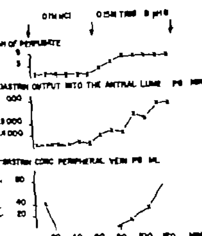
Discussion

The aim of the present investigation was to study the effect of intraantral pH on basal gastrin release into the antral lumen and into the circulation. The intraluminal gastrin secretion has been described in detail elsewhere (Uvnäs-Wallensten 1977).

The data in this communication show that there is no simple relationship between intraantral pH and the basal release of gastrin. It was found that even during antral perfusion with 0.1 M HCl gastrin was released into the antral lumen (~1 500 pg/min). During a preceding as well as during a subsequent perfusion with 0.15 M NaCl (pH 6.8) similar amounts of gastrin were secreted into the lumen. Nor did perfusion with citrate buffer (pH 3 and 5) or with 0.15 M NaCl pH 4 subsequent to perfusion with 0.1 M HCl cause any appreciable increase of the gastrin output. These results indicate that within an intraantral pH range of 1–7 basal gastrin release is uninfluenced by variations in pH

On the other hand, when the antrum was perfused with slightly alkaline buffer solutions such as 0.1 M phosphate buffer pH 7.4 sodium bicarbonate or Tris buffer increased amounts of gastrin were released not only into the antral lumen, but also into the circulation—as evidenced by increased peripheral gastrin levels. Perfusion with 0.01 and 0.1 M NaOH also increased the release of gastrin.

Dragstedt and Woodward and their colleagues (1951 1954 and 1957) studied the effect of pH on the rate of gastric acid secretion in Pavlov pouch dogs, with the antrum in situ, and after its transplantation to the doudenum or col They found that the



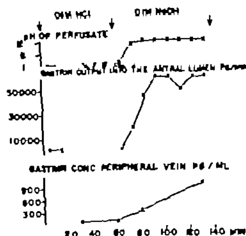


Fig. 2   Fig. 3

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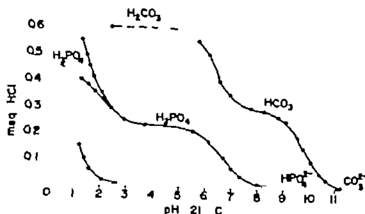


Fig. 4 Titration curves for NaCO and Na_2HPO ith 5 M HC Titration with 2 00 ml 0 15 NaCO. Titration ith 2 00 0.114 M Na_2HPO Titration w 2 00 ml H_2O. The lower titration curve for Na_2HPO demonstrat net values, *i e* total dues–d values obtained by titration wi 2 ml H_2O (the lowest curve)

factor (Fig. 4) Also perfusion with 0 1 M NaHCO and 0 1 M NaOH released gastrin, whereas perfusion with NaCl pH 8 was without effect These data indicate that in addition to HPO HCO ions and OH ions may stimulate the release of gastrin. Titration curves for Na_2CO and Na HPO are presented in Fig. 4

It should be stressed however that we cannot exclude that the increased gastrin release could be exerted by alkaline pH itself The stronger buffering capacity of phosphate buffer around pH 7 could in that case explain why antral perfusion with this buffer seemed to release more gastrin than other solutions of the same pH.

However if the gastrin release is induced by anions such as HPO HCO and OH where do these ions exert their stimulating effect? Since the antral G-cells have an apical part that projects into the lumen, receptors on the luminal cell surface might be activated It is also possible that the anions exert their effects intracellularly since they all penetrate cell membranes.

Is the gastrin release promoted by anions such as HPO HCO and OH of any physiological significance? It is a well known fact that aromatic amino acids and small peptides are only poor gastrin releasers (Debas *et al* 1974) In the present experiments even 0 001 M solutions of phosphate buffer stimulated the release of gastrin. Food (*e.g* meat and fish) contains substantial amounts of phosphate, the concentration of phosphate ions in *e.g* commercial meat broth being 0 005 M These ions may well contribute to the gastrin release caused by a meal In this context observations made by Öbrink (1954) are of interest. He found that the gastric secretion in dogs, caused by a bone dust – meat mixture-test meal, could be solely attributed to its phosphate content The effect might habe been mediated via a release of gastrin

The gastrin release induced by anions such as HCO might play a role under certain pathological conditions. In the intact empty stomach the pH in the antrum content is controlled by two factors, the alkaline bicarbonate secretion emanating from epithelial cells of the antral mucosa (Grossman 1959 McLeay and Titchen 1975 Flemström 1977) and the HCl secretion emanating from the parietal cells of the corpus. Normally the acid secretion dominates quantitatively rendering the pH of the antral pH more or less acidic. In the absence of HCl secretion the situation may be different When no acid neutralizes the antral alkaline secretion the pH of the mucosa will rise to 7–8 Thus in patients with atrophic gastritis, which have an intact antral mucosa, the pH may rise to ~8. The

...tration is in fact quite similar to that in the present experimental studies in which the ...olated antrum is perfused with phosphate or bicarbonate buffers. It is thus possible that ...e high gastrin plasma levels observed in patients with achlorhydria (McGuigan and ...rudeau 1970 Yalow and Berson 1970, Ganguli *et al* 1971 Stadil and Rehfeld 1973 and ...alenkrug 1975) may at least in part be due to an intense activation of the gastrin ...lease caused by endogenous secretion of bicarbonate.

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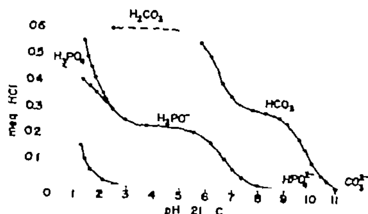


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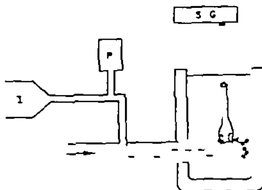


Fig. 1. Experimental set-up for recording of longitudinal tension and resistance to flow through the isolated, perfused bladder-urethral preparation. I, infusion pump, P, pressure transducer, S-G, strain-gauge transducer. For further details, see text. At the arrow the organ bath is supplied with pre-heated, oxygenated Krebs solution.

not only on resistance to flow through the practically closed urethra, but also on longitudinal tension. The aim of the present study was to investigate, by use of this technique, the effects of acetylcholine, noradrenaline, and prostaglandins on resistance to flow in the isolated, human fetal urethra. In addition, the occurrence of adrenergic nerve terminals in the fetal bladder, bladder neck, and urethra were investigated by means of the fluorescence method of Falck and Hillarp.

Materials and methods

Tissue specimens

Fetal bladder and urethral tissue was obtained at legal interruptions of pregnancy performed by hysterotomy. The estimated gestational age of the fetuses ranged from 16 to 22 weeks. Nine fetuses, 4 female and 5 male, were investigated.

Preparation and mounting

Approximately 10 mm of the urethra attached to the bladder was carefully dissected. The bladder base and a small part of the bladder wall were allowed to remain, and were tied to a PE 190 catheter. The bladder wall tissue was used to secure the portion of the catheter whose tip was placed just before the internal urethral orifice without stretching or occluding this structure. Through the catheter, which was connected to a perfusion pump (Braun perfusor), the bladder neck and proximal urethra were perfused at a constant rate of 2 ml/h with pre-heated (37°C) Krebs solution. The recorded resistance to flow was exerted largely by the urethra (Persson and Andersson 1976). The Krebs solution had the following composition (mM): NaCl 118, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.15, $NaHCO_3$ 24.9, KH_2PO_4 1.15, glucose 5.5, pH 7.4.

The preparation was mounted in a thermostatically controlled 50 ml organ bath (Fig. 1) containing Krebs solution (37°C) bubbled with 95 % O_2 and 5 % CO_2. The distal end of the urethra was, without occluding the lumen, tied to a strain-gauge transducer (Statham Ft 03) for recording of longitudinal isometric tension changes (Fig. 1). These recordings thus reflected changes in the urethral smooth muscle tone with a minor contribution of the bladder neck (*cf.* Persson and Andersson 1976). Basal tension was set at 3–5 mN depending on the size of the preparations.

Detrusor muscle strips were dissected into preparations being approximately 1 mm wide, 5 mm long and 0.5 mm thick. The apex and the trigonal area of the bladder were cut off and the remaining ring of detrusor muscle was mounted as such, or as a longitudinal strip after being cut open.

Histochemical analysis

Tissue specimens for histochemical study were taken from 4 of the fetuses, 2 male and 2 female. Along the longitudinal extensions, the urinary bladders and the urethrae were cut into pieces, equal in length and comprising the whole circumference of the respective organs. The tissue pieces were quickly frozen in a mixture of propane and propylene at the temperature of liquid nitrogen, freeze-dried and further processed for the Falck-Hillarp technique to demonstrate adrenergic nerves (*cf.* Björklund *et al.* 1972).

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From the Department of Clinical Pharmacology Institute of Pharmacology University of Aarhus, Denmark, the Research and Development Laboratories, AB Draco, Lund, the Institute of Anatomy and Histology University of Lund, and the Department of Obstetrics and Gynaecology University Hospital of Malmö, Sweden

Effects of acetylcholine, noradrenaline, and prostaglandins on the isolated, perfused human fetal urethra

By

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Abstract

Andersson K E., C G A Persson P Alm, S Kullander and U Ulmsten, *Effects of acetylcholine noradrenaline and prostaglandins on the isolated, perfused human fetal urethra* Acta physiol scand. 1978 *104* 394–401

Preparations of the isolated bladder-urethral junction obtained from human fetuses at midgestation were suspended in Krebs solution (37°C), and the urethral lumen was perfused at a low rate (2 ml/h) Resistance to flow and changes in longitudinal tension were recorded. In additional experiments, isometric tension changes were recorded in isolated detrusor strips. The occurrence of adrenergic nerve terminals was investigated by the fluorescence technique of Falck and Hillarp. Acetylcholine (0 1 1.0 μg/ml) produced concentration-related increase in longitudinal tension and in resistance to flow the effects were unaffected by phenoxybenzamine (0 1 μg/ml), but completely inhibited by atropine (0 1 μg/ml). The effect on tension was more marked than that on resistance to flow Noradrenaline (0 005–1.0 μg/ml) also produced concentration related increase in longitudinal tension and in resistance to flow the effects were abolished by phenoxybenzamine (0.1 μg/ml). The amine had a more pronounced effect on resistance to flow than on longitudinal tension. Prostaglandin $F_{2\alpha}$ (0 1–0 5 μg/ml) increased longitudinal tension and resistance to flow the effects were not antagonized by atropine (0 1 μg/ml) nor by phenoxybenzamine (0.1 μg/ml) In preparations contracted by noradrenaline or acetylcholine prostaglandins E and E_2 (0.02–0 2 μg/ml) decreased resistance to flow and longitudinal tension. Acetylcholine (0.02–0.2 μg/ml) and prostaglandin $F_{2\alpha}$ (0.0–0.5 μg/ml) contracted, noradrenaline (0 05–0 5 μg/ml) relaxed, and prostaglandin E and E_2 (0.1 5.0 μg/ml) had no consistent effects on the detrusor strips. Histochemically no adrenergic nerve terminals could be demonstrated in the detrusor bladder neck or urethra.

In isolated strips of smooth muscle from the human detrusor bladder neck, and urethra, contracting and relaxant effects of drugs acting on cholino- and adrenoceptors (Todd and Mack 1969 Nergårdh and Boréus 1972, Awad *et al* 1974 Caine *et al* 1975, Sundin *et al*, 1976 Ek *et al* 1977 b) and of prostaglandins (Abrams and Feneley 1976, Bultitude *et al*. 1976, Andersson *et al* 1977) have been demonstrated. However an *in vitro* analysis of such effects on the resistance to flow through the human urethra has not been possible.

In a previous investigation on the isolated bladder-urethral junction of cats and guinea pigs (Persson and Andersson 1976), a perfusion technique was used providing information

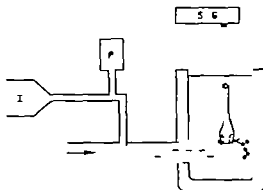


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Fetal bladder and urethral tissue was obtained at legal interruptions of pregnancy performed by hysterotomy. The estimated gestational age of the fetuses ranged from 16 to 22 weeks. Nine fetuses, 4 female and 5 male, were investigated.

Preparation and mounting

Approximately 10 mm of the urethra attached to the bladder was carefully dissected. The bladder base and a small part of the bladder wall were allowed to remain, and were tied to a PE 190 catheter. The bladder wall tissue was used to secure the position of the catheter whose tip was placed just before the internal urethral orifice without stretching or occluding this structure. Through the catheter, which was connected to a perfusion pump (Braun perfusor), the bladder neck and proximal urethra were perfused at a constant rate of 2 ml/h with pre-heated (37°C) Krebs solution. The recorded resistance to flow was exerted largely by the urethra (Persson and Andersson 1976). The Krebs solution had the following composition (mM): NaCl 118, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.15, $NaHCO_3$ 24.9, KH_2PO_4 1.15, glucose 5.5, pH 7.4.

The preparation was mounted in a thermostatically controlled 50 ml organ bath (Fig. 1) containing Krebs solution (37°C) bubbled with 95% O_2 and 5% CO_2. The distal end of the urethra was, without occluding the lumen, tied to a strain-gauge transducer (Statham F 03) for recording of longitudinal isometric tension changes (Fig. 1). These recordings thus reflected changes in the urethral smooth muscle tone with a minor contribution of the bladder neck (*cf.* Persson and Andersson 1976). Basal tension was set at 3 to 5 mN depending on the size of the preparation.

Detrusor muscle strips were dissected into preparations being approximately 1 mm wide, 5 mm long and 0.5 mm thick. The apex and the trigonal area of the bladder were cut off and the remaining ring of detrusor muscle was mounted as such, or as a longitudinal strip after being cut open.

Histochemical analysis

Tissue specimens for histochemical study were taken from 4 of the fetuses, 2 male and 2 female. Along the longitudinal extension, the urinary bladders and the urethras were cut into pieces, equal in length and comprising the whole circumference of the respective organs. The tissue pieces were quickly frozen in a mixture of propane and propylene at the temperature of liquid nitrogen, freeze-dried and further processed for the Falck-Hillarp technique to demonstrate adrenergic nerves (*cf.* Björklund *et al.* 1972).

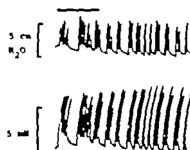


Fig. 2. Spontaneous activity of the fetal bladder-urethral junction. Rhythmic changes in isometric tension (lower tracing) were accompanied by corresponding fluctuations in resistance to flow (upper tracing). Horizontal marker: 4 min.

From one male fetus, tissue pieces were, after the freeze-drying, processed for the immunochemical demonstration of peptidergic (VIP: vasoactive intestinal polypeptide) nerves as described by Larsson et al. (1976).

Drugs

Drugs used were (−) noradrenaline bitartrate, (±) isoprenaline hydrochloride (Sigma Chemical Company, USA), phenoxybenzamine hydrochloride (Smith, Kline and French, UK), acetylcholine chloride (Calbiochem, USA), atropine sulphate (Pharmacopea Nordica), prostaglandin $F_{2\alpha}$, prostaglandin E_1, prostaglandin E_2 (AB ASTRA, Sweden). The drugs were injected directly into the bath. Concentrations given are final bath concentrations of active substance.

Results

The perfused fetal human urethra showed a good viability *in vitro*. During the first hour after mounting, the preparations developed a spontaneous contractile activity. Rhythmic changes in isometric tension were accompanied by corresponding fluctuations in resistance to flow (Fig. 2). The spontaneous activity decreased successively and lasted for 1–3 h. Basal tension was stable during the experiment. The basal resistance to flow was also stable, and varied between preparations from 5 to 12 cm H_2O.

Acetylcholine (0.1–1.0 μg/ml) produced a concentration-related increase in approximate longitudinal tension and in resistance to flow. Particularly at high concentrations, the drug induced a marked rhythmic contractile activity (Fig. 3). In three of the five preparations investigated, marked effects on tension were accompanied by less conspicuous effects on resistance to flow. The effects of acetylcholine were not inhibited by phenoxybenzamine (0.1 μg/ml), but were completely prevented if atropine (0.1 μg/ml) was present in the bath (Fig. 3).

In four out of five preparations, noradrenaline (0.005–1.0 μg/ml) produced a concentration-related increase in longitudinal tension and in resistance to flow (Fig. 4). Noradrenaline also produced a rhythmic contractile activity. The effects were more sustained than those produced by acetylcholine. It was consistently observed that noradrenaline augmented resistance to flow more than it increased longitudinal tension.

Phenoxybenzamine (0.1 μg/ml) left in contact with the preparation for 20 min, inhibited the noradrenaline effects. In one preparation, no contracting effect of noradrenaline was observed. However, the drug had a slight relaxant action when the preparation was contracted by acetylcholine.

In three preparations contracted by either acetylcholine or noradrenaline, the effects of prostaglandins E_1 (PGE$_1$) and E_2 (PGE$_2$), 0.02–0.2 μg/ml, were ... Consistent

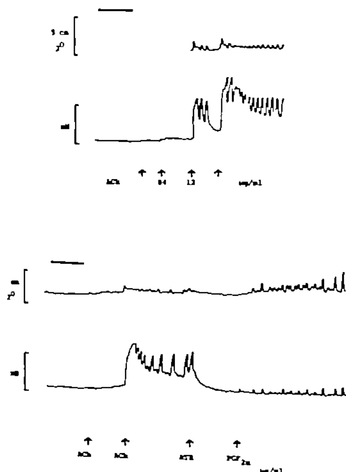


Fig. 1. Above: Effect of cumulating concentrations of acetylcholine (ACh) on longitudinal tension (lower tracing) and on resistance to flow (upper tracing) in an isolated bladder-urethral preparation. Below: Tracings as above. The effects of acetylcholine are abolished when atropine (ATR) is added to the bath. Atropine does not prevent the contractile effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Horizontal marker: 4 min.

inhibitory effects, i.e., a decrease in resistance to flow and in longitudinal tension, were produced by PGE (Fig. 4). PGE_1 mimicked the actions of PGE_2, except in one preparation, where no effect was registered.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 0.1–0.5 μg/ml, contracted the preparations, increasing the resistance to flow and longitudinal tension (Fig. 3). The effects of $PGF_{2\alpha}$ were not antagonized by atropine (0.1 μg/ml) and/or phenoxybenzamine (0.1 μg/ml).

The detrusor strips (n = 5) exhibited spontaneous contractile activity which was present during the first 2 h after mounting. Acetylcholine (0.02–1.0 μg/ml) and $PGF_{2\alpha}$ (0.1–0.5 μg/ml) consistently contracted the detrusor preparations and produced a rhythmic contractile activity (Fig. 5). Noradrenaline (0.05–0.5 μg/ml) inhibited spontaneous (Fig. 5), as well as drug-induced activity. PGE and PGE_2 (0.1–5.0 μg/ml) were without consistent effects on the detrusor preparations.

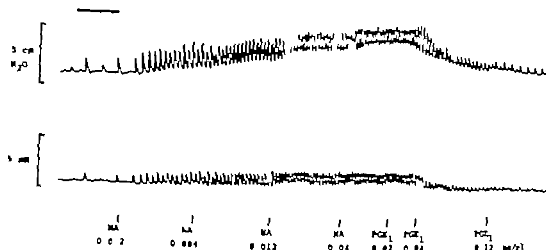


Fig. 4. Cumulating concentrations of noradrenaline (NA) increase resistance to flow (upper tracing) and longitudinal tension (lower tracing) in the bladder-urethral preparation. Prostaglandin E (PGE₁) added in the presence of noradrenaline produces inhibitory effects. Horizontal marker: 4 min.

Histochemical analysis

Despite serial sectioning, no specific fluorescence for adrenergic nerves or adrenergic ganglion cells could be demonstrated in any part of the urinary bladder and urethra (including the trigonal area and bladder neck), neither in the male nor in the female fetuses. Further, it was not possible in these organs to demonstrate the immunochemical presence of VIP nerves or VIP ganglion cells.

Discussion

Several previous studies have focused on the distribution of adreno- and cholinoceptors in the human bladder, bladder neck, and urethra. It has been clearly demonstrated that the detrusor and the bladder neck are contracted by cholinoceptor stimulating drugs, and that this effect can be blocked by atropine (Todd and Mack 1969, Caine *et al.* 1975). It is also generally agreed that the bladder neck contains mainly contraction-mediating α-adrenoceptors, whereas the detrusor has a predominance of relaxation-mediating β-adrenoceptors (Todd and Mack 1969, Nergårdh and Boréus 1972, Awad *et al.* 1974, Sundin *et al.* 1977).

Ek *et al.* (1977 b), demonstrated that preparations from all parts of the isolated human urethra, including the urethro-vesical junction, were contracted by noradrenaline in a concentration related way. Acetylcholine consistently contracted the urethro-vesical junction, but in all other parts of the urethra, the sensitivity to the drug was low, and often no effects were observed. These *in vitro* results were in agreement with investigations *in vivo*, showing that the intra-urethral pressure can be increased by α-adrenoceptor stimulating drugs (Schreiter *et al.* 1976, Ek *et al.* 1977 c), and decreased by α-adrenoceptor blocking agents (Donker *et al.* 1972, Awad *et al.* 1976, Whitfield *et al.* 1976, Ek *et al.* 1977 c, Sundin *et al.* 1977). Further, they were consonant with the findings that the *in vitro*

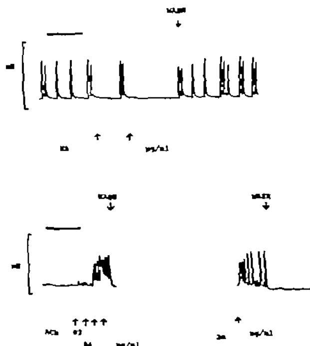


Fig. 5 Upper tracing: Noradrenaline (NA) inhibits the spontaneously active fetal bladder strip. After wash out the contractile activity returns. Lower tracing: Acetylcholine (ACh) and prostaglandin $F_{2\alpha}$ ($F_{2\alpha}$) contract and induce rhythmic activity in the bladder strip preparation. Horizontal marker: 4 min.

effect of cholinoceptor stimulation by bethanecol on the urethral closure pressure profile was negligible (Ek *et al.* 1977 c), and that cholinoceptor blockade by emepronium bromide was …hout effect on this parameter (Ulmsten and Andersson 1977).

In the investigations on the isolated human urethra, (Ek *et al.* 1977 b), the activity of the circular muscle layer was recorded. The technique used in a previous study on the isolated, perfused urethra of cat and guinea-pig (Persson and Andersson 1976) made it possible to measure simultaneously circular muscle activity as reflected by resistance to flow and longitudinal muscle activity registered as changed in isometric tension. Using this technique, it could be shown that in cat and guinea-pig, acetylcholine had less effect on resistance to flow than on longitudinal tension. Furthermore, noradrenaline was more effective than acetylcholine in contracting the circular muscle of the urethra (Persson and Andersson 1976).

The present results agree well with those obtained with the perfused animal urethras. It was found that noradrenaline contracted the isolated human fetal urethra in a concentration-related way increasing both resistance to flow and longitudinal tension. The effect on resistance to flow was the more pronounced, probably reflecting a more marked effect on the circular than on the longitudinal muscle layer. Acetylcholine also had a concentration-related contracting action. The effect was less well sustained than that produced by noradrenaline, and longitudinal tension was often more affected than resistance to flow. This suggests that acetylcholine had a more pronounced effect on the longitudinal muscle layer than on the circular.

Thus, the results seem to give further support to the view that acetylcholine is of less importance than noradrenaline for maintaining urethral wall tension in man, as this is probably determined mainly by activity in the circular muscle layer

It was previously shown that prostaglandins E and E_2 decreased resistance to flow through the isolated perfused feline urethra (Persson 1976) *In vitro* these prostaglandins contracted the bladder but relaxed the urethral smooth muscle of human adults (Andersson *et al* 1977) The effects could be reproduced *in vivo* locally applied prostaglandin E_2 increased the intravesical pressure and decreased the intraurethral pressure in women (Andersson *et al* 1978) The present study showed that prostaglandins E and E_2 had no consistent effects on the detrusor preparations, but decreased resistance to flow and longitudinal tension also in the isolated, human fetal urethra.

Adrenergic nerve terminals have been demonstrated in the human detrusor bladder neck and urethra of adults (Mobley *et al* 1966, Sundin *et al* 1977 Ek *et al* 1977 a). It was found that in the detrusor and urethra, the smooth muscle cells had a scanty supply of adrenergic nerves, but in the trigone area, the adrenergic nerves were abundant. Gosling and Dixon (1976), and Nordling and Christensen (1977) found a very sparse noradrenergic nerve supply in "non-neurogenic human bladders" and the latter authors concluded that this innervation hardly could be of physiological significance. In the present study no adrenergic nerve terminals could be demonstrated, neither in the bladder and urethra, nor in the trigone area This might be explained by a slow development together with a scanty occurrence of such nerve terminals in the investigated structures

Despite the absence of adrenergic nerves, well developed responses to noradrenaline were demonstrated Thus, the distribution and function of adrenoceptors did not seem to be correlated to the occurrence of adrenergic nerve terminals. This is in agreement with the results of other investigators (see, Pappano 1977) In the human fetal heart adrenergic nerve terminals cannot be demonstrated histochemically before the 12th week of gestation (Kanerva *et al* 1974) but a well developed response to adrenaline was found already in the 9th gestational week (Gennser and Nilsson 1970)

Recently the occurrence of nerves containing vasoactive intestinal peptide (VIP) was shown in the genito-urinary tract of pigs, cats, rats, and mice (Alm *et al* 1977 Larsson *et al* 1977 a, b) No such nerves were found in the fetus examined in the present study However as judged from preliminary results, VIP nerves can be demonstrated in the adult human urinary bladder (Alm unpublished observations)

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Metabolic and cardiovascular responses to prolonged noradrenaline load and their antagonism by beta blockade in the rat

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Abstract

HARRI M N E. *Metabolic and cardiovascular responses to prolonged noradrenaline load and their antagonism by beta blockade in the rat* Acta physiol scand 1978 *104* 402-414

Rats were given daily injections of noradrenaline (0.5 mg/kg), alprenolol (20 mg/kg), or both together for 17 to 39 days. Noradrenaline injections increased the size of the heart, interscapular brow adipose tissue (ISBAT) and, when the i jections were given at a thermoneutral environment, the metabolic activity of the ISBAT Alprenolol i jections tended to reduce the size of the heart and ISBAT and, t a thermoneutral environment metabolic activity f the skeletal muscle Furthermore noradrenaline treatment improved tolerance against body cooling in water at 25°C and enhanced calorigenic response to injected noradrenaline The metabolic alterations in the rats injected with both drugs together resembled more those of the animals injected with alprenolol only than those of the noradrenaline treated rats. It can be thus concluded that alprenol l, when given together with noradrenaline antagonized noradrenaline-induced metabolic alterations in the organism. However the increased pressor response to infused noradrenaline, which was evident in the alprenolol treated animals, was not seen in the animals treated with both drugs. By co trast these nimals, like also the noradrenaline-treated animals, showed decreased pressor response to noradrenaline This f nding suggests that prolonged beta blockade does not abolish the decreased sensitivity of alpha-adrenoreceptors following prolonged noradrenaline load. On the other hand, the hypotonic response to beta adrenergic drug, isoprenaline was reduced by prolonged beta blockade despit simultaneous noradrenaline injections.

Key words: long-term beta blockade nzymatic changes, brown adipose tissue, cold tolerance non-shivering thermogenesis, adrenergic responses

Activation of the sympathetic nervous system is known to occur in exposure and acclimation to cold This activation can be simulated by repeated injections of noradrenaline to such an extent that noradrenaline-treated rats show a larger oxygen consumption and rectal temperature increases at cold, an increased resistance to cold, and an enlargement of brown adipose tissue (LeBlanc and Poulliot 1964 LeBlanc and Villemaire 1970, Vallieres *et al.* 1972, Hsieh and Wang 1971) This treatment, like cold acclimation, also results in enhanced sensitivity to injected catecholamines (LeBlanc and Villemaire 1970 Vallieres *et al* 1972, Hsieh and Wang 1971), which has been interpreted to be due to increased sensitivity of adrenergic beta-receptors. Furthermore cold-acclimated and catecholamine-

tested rats have a decreased sensitivity to alpha-adrenergic drugs (Harri *et al.* 1974, Tirri *et al.* 1976). On the other hand, long-term pretreatment of rats with beta-blockers or chemical sympathectomy with 6-hydroxy dopamine lead to changes in metabolic and cardiovascular parameters and adrenoreceptor properties (Harri 1977a, Behrens and Depocas 1977, Krakoff and Ginsburg 1973), which are principally opposite to those resulting from cold-acclimation or repeated catecholamine injections (Harri and Valtola 1975).

Since beta blockers antagonize many of the acute effects of injected noradrenaline in the organism it was of interest to study whether or not prolonged beta blockade can antagonize the development of the metabolic and cardiovascular alterations following repeated noradrenaline injections. In a previous study it was shown that long-term beta blockade failed to antagonize the metabolic and adrenergic sensitivity alterations due to hyperthyroidism (Harri 1978), in spite of the fact that beta blockers have been employed with satisfactory results in the treatment of the many clinical manifestations of hyperthyroidism (Riddle and Schwarz 1978, Grossman *et al.* 1971 a, b) and that beta blockers reduce the metabolic responses to exercise in hyperthyroid dogs (Kaciuba Uściłko *et al.* 1976). To reduce the influence of increased release of endogenous catecholamines resulting from diminished thermoregulatory capacity due to beta-blockade (Helm and Hull 1966, Harri 1968, Irving *et al.* 1974, Banet and Hensel 1977), a group of rats was also maintained at a thermoneutral temperature during the drug treatment.

Material and methods

Animal care and treatment. Male rats of Wistar/Af/Han/(Han 67) strain initially weighing approximately 150 g were housed in groups of 4 and maintained on diet of standard laboratory chow (Hankkija, Finland) and water.

The first group was composed of control animals, injected daily with 0.3 ml of physiological saline glycerol mixture (3 : 2); the second group of rats injected with 0.5 mg noradrenaline/kg (l-noradrenaline bitartrate, Fluka AG, the dose is calculated as base) (group N), the third group of rats injected with 20 mg alprenolol hydrochloride/kg (AB Hässle) (group A) and finally the fourth group of rats injected with 20 mg alprenolol 0.5 mg noradrenaline/kg (group A+N). The drugs were given subcutaneously in saline-glycerol (3 : 2) mixture five times per week for the total number of injections indicated below. These animals were kept at room temperature (19–21°C) during the drug regime. In the second series, the rats also were treated with drug or control injections, but were maintained at thermoneutral temperature (28 °C) during the injection periods.

Measurements on unanaesthetized animals. 24 h after the 17th injection the cooling rate was measured for the rats which were swimming in water at 25°C (Dawson *et al.* 1970, Harri 1978). The colonic temperature response to injected noradrenaline (0.5 mg/kg i.p.) was determined at thermoneutral temperature of 29°C 24 h after the 19th injection (LeBlanc and Villemaire 1970, Harri 1978). Colonic temperatures were obtained with thermocouple inserted to depth of 4 cm, and recorded on an Ellab TE 3 (Copenhagen) potentiometer.

Cardiovascular responses. The heart rate and blood pressure responses to beta-adrenergic drug, isoprenaline, and to an alpha-adrenergic drug, noradrenaline was measured 24 h after the 31st to 39th injection. The animals were anaesthetized by veterinary Nembutal (pentobarbital sodium, Abbot), 50 mg/kg injected intraperitoneally. One carotid artery was cannulated for blood pressure measurement and the opposite jugular vein for the drug infusion. The body temperature of the animals was maintained at 37 ± 1°C with the aid of heat lamp. Continuous measurements of heart rate and blood pressure were made with strain gauge transducer (Hewlett Packard 267BC series), amplified with carrier amplifier (Hewlett Packard 8805B), and recorded on paper by ink-jet writing recorder (Elema, Schönander Mingograph 34).

Enzyme assays. The same rats which were used for these studies were not subjected to the above mentioned tests. The rats were killed 24 h after the 24th injection by decapitation. Heart, adrenals and

TABLE I Effect of daily (for 24–25 days) injections of alprenolol (20 mg/kg), noradrenaline (0.5 mg/kg) and their combination on body and organ weights and on relative density of nuclei in histologic sections of ISBAT The rats were housed at room temperature (19–21°C) during the injections

	Control (18)	Alprenolol (4)	Noradrenaline (10)	Noradrenaline + Alprenolol (10)
Δ Body weight g	102±5.3	95±6.4	102±3.0	93±3.0
Heart, mg/100 g	253±4.0	234±7.0	285±4.8	275±5.5
Adrenals, mg/100 g	21.5±0.33	24.3±0.84	24.5±0.96	25.0±1.23
ISBAT mg/100 g	113±5.9	88±5.7	166±16.5	128±6.5
Nuclei/mm in ISBAT	2 302±99	3 011±235	1 827±151	2 029±177

ISBAT = interscapular brown adipose tissue Number of rats in parentheses. Significant difference f the controls $p < 0.05$ $p < 0.01$ and $p < 0.001$ (Student's t test).

interscapular brown adipose tissue (ISBAT) were removed and weighed. Samples of the tip of the myocardium, of the thigh muscle (M astus lateralis) and of the ISBAT were immediately frozen in liquid nitrogen, and stored at –80 C until assayed The tissue samples were homogenized in a Potter Elvehjem glass homogenizer in Tris-HCl buffer (0 1 M pH 7 6) to give a 2% (w/v) homogenate and centrifuged for 10 min at 1 000 g t 4 C to remove broken cells and particulate debris. The supernatants were used for the determination of the activities of succinate dehydrogenase (SDH) (EC 1.3.99 1) (Earl and Korner 1965), malate d hydrogenase (MDH) (EC 1 1 1 37) (Englard and Siegel 1969), citrat synthase (CS) (EC 4 1 3 7) (Srere 1962), and hex kinase (HK) (EC 2.7 1 1) (Bass *et al.* 1969). The activity of lactate dehydrogenase (LDH) (EC 1 1 1.27) was determined usi g commercial reagents (Boehringer Mannheim) t Measurements wer made at 37°C w th a Cary 118 doublebeam spectrophotometer The assays of the four animal groups were always performed together The protein concentration of homogenates as estimated by the phenol method with bovine serum albumin as the standard (Lowry *et al.* 1951).

For the determination of lipid content, samples of ISBAT were dried at 100°C for 2 days, and homogenized in chloroform-methanol (2 1 /v) m ture. The total l pid content was then analyzed according to the method of Frings and Dun (1970).

Histological studies Samples of ISBAT were fixed in 10 per cent formol. After dehydration ith alcohol and embedding i paraffin, the 7 μm sections were stained with hematoxylin-eosin. The density of fat cell nuclei was calculated from photomicrographs.

Results

Body and organ weights The results in Table I show that the weight gain was identical in all four experimental groups when the animals were housed at a room temperature during the experimental period. Nordarenaline injections increased the relative size of the myocardium This increase was almost as pronounced in rats having received alprenolol injections in addition to noradrenaline as in the animals injected with noradrenaline only The size of the adrenals increased in response to the drug treatments used. However the relative size of ISBAT was increased only in N treated rats. By contrast, a significant reduction in the density of fat cell nuclei was noted in histological sections of this organ in these animals. In the A treated group changes in the size and density of fat cell nuclei of ISBAT were the opposite to those found in N-treated group, while these parameters in A + N-rats did not differ significantly from the controls.

The results from the series of experiments carried out at 28–29 C are presented in Table II At this temperature the weight gain was significantly lower in N-treated rats than in the

Table II. Effect of daily (for 4–25 days) injections of alprenolol (0.2 mg/kg), noradrenaline (0.5 mg/kg) and their combination on body and organ weights and on relative density of nuclei in histological sections of ISBAT. The rats were housed at thermoneutral temperature (28–29°C) during the injections

	Control (10)	Alprenolol (10)	Noradrenaline (10)	Noradrenaline + Alprenolol (10)
Body weight, g	102 ± 2.1	107 ± 3.4	96 ± 2.5	97 ± 2.5
Heart, mg/100 g	299 ± 3.5	297 ± 5.3	327 ± 4.3	315 ± 3.6
Adrenals, mg/100 g	21.6 ± 1.06	22.5 ± 1.05	28.4 ± 1.24	28.0 ± 0.92
ISBAT mg/100 g	112 ± 7.3	83 ± 3.9	118 ± 8.0	71 ± 5.2
Nuclei mm² in ISBAT	1 376 ± 123	1 890 ± 156	1 985 ± 139	1 874 ± 74

ISBAT interscapular brown adipose tissue. Number of rats in parentheses. Significant difference from the controls: p 0.05, p 0.01 and p < 0.001 (Student t-test).

atrols. The relative size of the heart was increased in N-treated and A + N-treated rats. The increase was, however significantly less in the A + N group ($p < 0.05$). The enlargement of the adrenals was significantly greater in the animal groups injected with noradrenaline alone or in combination with alprenolol than in the animals having received alprenolol injections only (p 0.001). The relative size of the ISBAT was smaller in the A and A + N-treated groups than in the control or N-group. The density of fat cell nuclei was, however significantly increased in all drug-treated animal groups.

Enzymatic changes. The series of experiments performed in conventional way at room temperature revealed no significant changes in cardiac or skeletal muscle enzyme activities (Table III).

In the ISBAT on the other hand, the activities of oxidative enzymes, SDH, MDH and CS, tended to be low in the N-treated animals and high in the A or A + N-treated groups. The activity of SDH was significantly higher in the alprenolol group than in the N-group (p 0.05), while the activity of CS was significantly higher both in the A ($p < 0.001$) and in the A + N-group (p 0.001) than in the animals having received N-injections only. Although a similar trend was observable in the activity of MDH, this trend did not attain a level of statistical significance. It is interesting to note that the activity of LDH, when expressed per unit tissue wet weight, was not decreased in the ISBAT of the N-animals, but showed an opposite trend. This is rather surprising because the protein (enzyme) concentration in the cell free tissue homogenate, which was used in the enzyme determinations, was significantly lower in this experimental group as compared to all other groups ($p < 0.01$–0.001). The activity of HK of ISBAT was so low that it cannot be used as an anaerobic marker enzyme for this tissue.

The decreased protein concentration of the ISBAT homogenate in N-treated animals and a similar trend in A N-treated animals is coupled with the increased amount of lipids in ISBATs of these both groups and, in addition, with the increased water content in the ISBAT of N-treated animals. It should be kept in mind that the protein concentration was determined from the 1 000 g supernatants, not from the crude homogenate. Therefore it does not represent the total protein content of the tissue.

TABLE III Effect of daily (for 24-25 days) injections of alprenolol (20 mg/kg), noradrenaline (0.5 mg/kg) and their combination on enzyme activities and protein concentration of rat tissues and on water and lipid concentration of ISBAT The rats were housed at room temperature (19-21°C) during the injections.

	Control	Alprenolol	Noradrenaline	Noradrenaline + Alprenolol
Myocardium				
Succinate dehydrogenase	39 1±1 01	37.3±1 76	38.1±0.76	36.3±0 69
Malate dehydrogenase	679±12.5	696±35.9	706±15.4	658±15.3
Citrate synthase	146±2.37	144±1 17	148±3 15	141±2.99
Hexokinase	3 42±0.162	3.02±0.240	2.99±0.231	3 03±0.156
Lactate dehydrogenase	941± 1.0	872±16.1	891±23.0	862±17 6
Protein	88 5±1.05	89.0±1.25	85.0±3 13	84 7±1 76
M vastus lateralis				
Succinate dehydrogenase	7 42±0.263	6.92±0.696	6.97±0.696	7 48±0 329
Malate dehydrogenase	198±5 0	189±13.2	187±8.1	192±6.1
Citrate synthase	26.2±0 51	26.0±1 19	24.6±1.40	24.4±0.93
Hexokinase	3.30±0.119	3 10±0.570	3.54±0.155	3.53±0.284
Lactate dehydrogenase	1 030±24 8	1 030±47.3	1 094±55 7	1 057±33.2
Protein	71 0±1 60	68.9±3 45	71.3±1.55	72.0±0.72
ISBAT				
Succinate dehydrogenase	23 4±1 17	23.2±1 76	18.4±1 15	1.2±1 17
Malate dehydrogenase	449±24 1	473±30.5	455±31.5	515±42.1
Citrate synthase	233±24 9	281±28 5	143±22.4	245±22.7
Hexokinase	1.39±0 208	—	1 76±0.234	1.22±0 169
Lactate dehydrogenase	254±27.2	260±10.7	332±47.5	269±22.8
Protein	73.8±3 00	79 5±2.85	50.3±4 55	63.9±6 05
Water %	34 6±2 03	35.1±0.85	41 7±2.50	35.0±1.33
Total lipids, % of dry wt.	57 3±1.25	61 1±2.94	63.2±1.56	66.2±0.68

ISBAT = Interscapular brown adipose tissue The number of rats is identical to those given in Table I Enzyme activities are expressed as μmoles of substrate utilized per min per g wet weight, and protein as mg in cell-free tissue homogenate from 1 g of tissue Significant difference from the controls. $p<0.05$, $p<0.01$ and $p<0.001$ (Student t-test).

The results in Table IV show that in the series of experiments carried out at a thermoneutral temperature the treatments used caused much more changes in the enzyme activities than was observable when the treatments were performed at a room temperature. In the myocardium, however there was rare significant changes. The only significant differences from the control group were the decreased activity of CS in the A + N-treated group and the increased activity of MDH in the A treated group Furthermore, the activity of SDH was significantly ($p<0.05$) lower in the A + N treated group as compared to the animals having received N injections only The protein content in the myocardial homogenate from the A-treated animal was, however significantly ($p<0.05$) higher as compared to N treated or A + N-treated rats. The enzyme activities of the skeletal muscle in N-treated group were identical to those in the controls. However the activities of oxidative enzymes, SDH MDH and CS in the A and A + N-groups were not only significantly lowered in comparison with the control animals but also significantly lowered in comparison with the N-group The only exception was the difference in MDH activity between A and N-groups

Table IV Effect of daily (for 24–25 days) injections of alprenolol (20 mg/kg), noradrenalin (0.5 mg/kg) and their combination on enzyme activities and protein concentration of rat tissues and on water and lipid concentration of ISBAT. The rats were housed at thermoneutral temperature (28–29°C) during the injections.

	Control	Alprenolol	Noradrenaline	Noradrenaline + Alprenolol
Myocardium				
Succinate dehydrogenase	37.2±1.06	38.7±0.98	38.0±0.84	35.3±0.73
Malate dehydrogenase	748±19.9	837±9.9*	766±12.9	742±11.9
Citrate synthase	138±1.67	141±2.11	137±2.43	131±2.42
Lactate dehydrogenase	878±40.8	888±17.8	881±32.5	913±16.7
Protein	82.2±2.32	84.9±0.83	81.9±0.98	81.9±0.88
M. vastus lateralis				
Succinate dehydrogenase	7.63±0.399	6.22±0.262	7.23±0.431	6.01±0.250
Malate dehydrogenase	208±10.4	165±5.8	182±8.7	153±4.2*
Citrate synthase	23.2±1.27	18.4±0.63	20.6±0.90	16.9±0.40
Lactate dehydrogenase	998±44.8	959±22.8	943±22.7	929±19.3
Protein	71.4±1.19	69.1±1.19	67.7±1.35	66.1±1.19
ISBAT				
Succinate dehydrogenase	14.0±1.73	24.6±1.98	27.4±1.58	23.7±1.93
Malate dehydrogenase	238±29.4	354±25.9	363±18.9	332±13.4
Citrate synthase	93±13.0	187±15.0*	190±11.3	154±11.4
Lactate dehydrogenase	303±46.1	301±18.8	419±29.9	331±20.0
Protein	38.2±1.81	74.2±3.31	68.7±3.46	66.8±2.52
Water	—	25.0±1.35	36.7±1.93	34.2±1.72
Total lipid, % of dry wt.	—	56.6±1.27	53.7±2.19	53.9±2.56

ISBAT, interscapular brown adipose tissue. The number of rats is identical to those given in Table II. Enzyme activities are expressed as μmoles of substrate utilized per min per g wet weight, and protein as mg in cell-free homogenate from 1 g of tissue. Significant difference from the controls: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ (Student's t-test).

which did not attain a level of statistical significance. The activity of LDH was identical in all four experimental groups.

The metabolic activity of the ISBAT was higher in drug-treated groups than in the controls, as judged from the elevated activity of oxidative enzymes in all groups and also of the LDH in the N-treated group. Moreover, the activity of CS was significantly lower in the A+N-group and that of LDH in the A- and A+N-groups in comparison to the animals having received N-injections only ($p < 0.05$). A similar trend, although statistically not significant, was observable in the activities of SDH and MDH, too. The protein content of the cell free homogenate of ISBAT was higher in drugtreated rats than in the controls but no differences were found between different drug treatments. Also the fat content of the ISBAT was identical for all drug-treated groups. The water content of the ISBAT was, however, greater in the N-treated and A+N-treated rats ($p < 0.001$) than in the animals injected with alprenolol only. A comparison with Table II reveals that the density of fat cell nuclei in histological sections of ISBAT is greater in drug-treated groups. Furthermore, the fat droplets in the control ISBAT were large and mostly unilocular in comparison with the multilocular appearance in the drug-treated rats.

TABLE III Effect of daily (for 24–25 days) injections of alprenolol (20 mg/kg), noradrenaline (0.5 m[illegible] and their combination on enzyme activities and protein concentration of rat tissues [illegible] water and lipid concentration of ISBAT The rats were housed at room temperature (1[illegible] during the injections.

	Control	Alprenolol	Noradrenaline	Noradrenaline + Alprenolol
Myocardium				
Succinate dehydrogenase	39 1 ± 1 01	37.3 ± 1 76	38.1 ± 0.76	36.3 ± 0 69
Malate dehydrogenase	679 ± 12.5	696 ± 35.9	706 ± 15.4	658 ± 15 3
Citrate synthase	146 ± 2.37	144 ± 1 17	148 ± 3.15	141 ± 2 99
Hexokinase	3 42 ± 0.162	3.02 ± 0.240	2.99 ± 0 231	3.03 ± 0.156
Lactate dehydrogenase	941 ± 21.0	872 ± 16.1	891 ± 23.0	86[illegible] ± 17.6
Protein	88.5 ± 1.05	89 0 ± 1.25	85.0 ± 3 13	84.7 ± 1.76
M vastus lateralis				
Succinate dehydrogenase	7 42 ± 0.263	6.92 ± 0.696	6.97 ± 0.696	7 40 ± 0.329
Malate dehydrogenase	198 ± 5 0	189 ± 13.2	187 ± 8.1	19[illegible] ± 6 1
Citrate synthase	26.2 ± 0.51	26 0 ± 1 19	24 6 ± 1 40	24 4 ± 0 93
Hexokinase	3.30 ± 0.119	3 10 ± 0.570	3.54 ± 0.155	3 53 ± 0 284
Lactate dehydrogenase	1 030 ± [illegible]4 8	1 030 ± 47.3	1 094 ± 55 7	1 057 ± 33.2
Protein	71 0 ± 1 60	68.9 ± 3 45	71.3 ± 1.55	72.0 ± 0.72
ISBAT				
Succinate dehydrogenase	23.4 ± 1 17	23.2 ± 1 76	18.4 ± 1 15	1[illegible] ± 1 17
Malate dehydrogenase	449 ± 24 1	473 ± 30.5	455 ± 31.5	515 ± 42.1
Citrate synthase	233 ± 4.9	281 ± 28.5	143 ± 22.4	[illegible]5 ± 22.7
Hexokinase	1 39 ± 0.208	—	1 76 ± 0.234	1.22 ± 0.169
Lactate dehydrogenase	[illegible]54 ± 27.2	260 ± 10 7	332 ± 47 5	269 ± 22 8
Protein	73.8 ± 3 00	79 5 ± 2.85	50.3 ± 4 55	63.9 ± 6 05
Water	34 6 ± 2.03	35 1 ± 0.85	41 7 ± [illegible].50	35.0 ± 1.35
Total lipids, % of dry wt.	57 3 ± 1.25	61 1 ± 2.94	63.2 ± 1.56	66.2 ± 0 68

ISBAT = Interscapular brown adipose tissue The numbe of rats is identical to those given in Table [illegible] Enzyme activities are expressed as μmoles of substrate utilized per min per g [illegible]et weight, and protein mg in cell-free tissue homogenat from 1 g of tissue Significant difference from the controls: p [illegible] $p < 0.01$ and $p < 0.001$ (Student s t-test).

The results in Table IV show that in the series of experiments carried out at a thermoneutral temperature the treatments used caused much more changes in the enzyme activit[illegible] than was observable when the treatments were performed at a room temperature In the myocardium, however there was rare significant changes. The only significant differences from the control group were the decreased activity of CS in the A + N treated group and the increased activity of MDH in the A treated group. Furthermore, the activity of SDH was significantly ($p < 0.05$) lower in the A + N-treated group as compared to the animals having received N injections only The protein content in the myocardial homogenate from the A-treated animal was, however significantly ($p < 0.05$) higher as compared to N treated or A + N treated rats. The enzyme activities of the skeletal muscle in N-treated group were identical to those in the controls. However the activities of oxidative enzymes, SDH MDH and CS, in the A and A + N-groups were not only significantly lowered in comparison with the control animals but also significantly lowered in comparison with the N-group. The only exception was the difference in MDH activity be[illegible] and N-groups

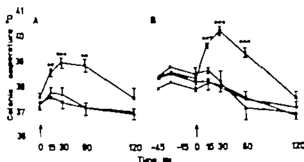


Fig. 2. The response of the colonic temperature to injected noradrenaline (0.5 mg/kg s.c.) at 28°C in control rats (Δ), in alprenolol-treated rats (20 mg/kg daily for 19 days) (●), in noradrenaline treated rats (0.5 mg/kg daily for 19 days (○), and in rats injected with alprenolol together with noradrenaline (x). A: the animals were maintained at 28–29°C during the injection periods and B: the animals were kept at room temperature. Vertical bars indicate ± S.E. Noradrenaline was injected at 0 min. Asterisks mark the hyperthermic changes which significantly differ from the initial values (0 min). ** $p < 0.01$ and *** $p < 0.001$ (Students' t-test). 4 rats per group.

of the previous treatment and in the animals maintained at a thermoneutral temperature, A+N pretreatment resulted in enhanced sensitivity to noradrenaline. This was seen by elevated colonic temperature after the test injection. In other experimental groups noradrenaline test injection failed to cause hyperthermia.

Fig. 3 shows the heart rate and blood pressure responses to infused noradrenaline and isoprenaline in anesthetized rats. This test was performed only on the rats, which were maintained at room temperature. The basic heart rates for the control, A, N- and A+N-pretreated groups were 435 ± 6.8, 407 ± 4.8, 409 ± 7.8 and 406 ± 8.9 beats/min, respectively. Thus the heart rates in all drug treated groups were significantly slower in comparison with the controls (p 0.02 to p 0.01). The initial systolic blood pressure values varied from the average value of 145 ± 4.8 mmHg in the controls to 159 ± 8.9 mmHg in the N-treated group, and the diastolic from 118 ± 3.8 to 124 ± 6.8 mmHg, respectively. No significant differences between various groups were found. The slight bradycardia caused by noradrenaline infusion was identical in all groups, while the tachycardic response to infused isoprenaline was significantly greater in the A- and N-treated animals than in the control or A+N-treated group. The pressor response to infused noradrenaline was significantly smaller in the N- and A+N-groups, while the rise of the diastolic blood pressure caused by noradrenaline was significantly greater in the A-treated animals. On the other hand, the hypotonic response to infused isoprenaline was identical in N-treated group as in the controls but significantly smaller in the A- and A+N-treated animals.

Discussion

In accordance with the findings of LeBlanc and Villemaire (1970) repeated injections of noradrenaline induced marked cardiomegaly in the rats. This effect was as marked in animals maintained at a room temperature during the treatment as in the animals maintained at thermoneutral temperature. It was only partially antagonized by simultaneous

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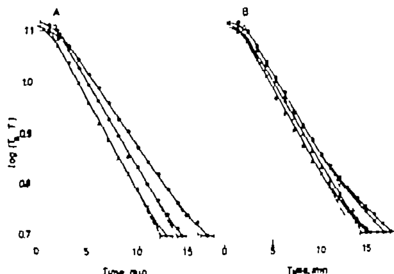


Fig. 1 The rate of body cooling in rats swimming in water at 25°C Δ = control rats, ● alprenolol treated rats (20 mg/kg daily fo 17 days), ○ = noradrenal ne treated rats (0.5 mg/kg daily for 17 days) and x = rats injected w th alprenolol together with noradrenaline A the animals ere maintained a 28–29 C during th i jection periode and B the animals were kept at a room temperature (19–21 C) $T_B - T_A$ = the difference of body and ambient (water) temperature The time of the body temperature to cool to 30°C is i dicated t the end of each line as the mean ± S.E. The broken lines extrapolate the initial rates of body cooling. 5 rat per group.

Whole animal experiments Fig. 1 shows the rate of cooling of rats swimming in water at 25 C In the series of experiments constructed at a room temperature the initial rate of body cooling was almost identical in all four experimental groups. The cooling constants for the initial phase of body temperature drop were calculated according to the formula of $C = 2.3\ d \log (T_B - T_A)/dt$ (Morrison and Tiez 1957) where T_B and T_A refer to body and ambient temperature respectively They were 4.33 ± 0 10 4 22 ± 0 16, 4.21 ± 0.17 and 4 48 ± 0 14 C/h for the control, N A and A N-groups, respectively These values do not differ significantly from each other However when the differences between ambient and body temperature becomes smaller in a living animal, the metabolic heat production slows down the rate of body cooling. This can be seen by a shift of the cooling rate curve to the right from the initial slope Owing to this shift, the time of the body temperature to cool to 30°C was significantly longer for the N or A treated groups than for the controls or A + N-treated group ($p < 0.05$ to p 0 01) being 17.9 ± 0 57 17 0 ± 0.54 15.5 ± 0.62 and 15 1 ± 0 71 min for the N A control and A + N groups, respectively

On the other hand in the series of experiments in which the animals were kept at 28–29 C during the drug injections, there was much marked differences between the various drug treated groups. The cooling constants, which were 4 89 ± 0 09 4.52 ± 0.18 and 3.91 ± 0 10°C/h for the A + N A and N-treated groups, respectively did differ significantly from each other ($p < 0.05$–$p < 0.01$). Furthermore, the times of the body temperature to cool to 30°C were 12.8 ± 0 65 14.5 ± 0 41 and 17 0 ± 0 68 min for the A + N A and N-treated groups, respectively they did differ significantly from each other ($p < 0.05$ to $p < 0.02$).

Fig. 2 shows the metabolic response of the rats to injected noradrenaline (0.5 mg/kg i.p.) when measured at 28 C. Both in the animals maintained at a room temperature during

is supported also by the results obtained from the series of experiments performed at a room temperature. At this environment the impaired thermoregulatory capacity due to beta blockade, results in a compensatory release of endogenous noradrenaline (*cf.* Harri 1977 a, b, Irving *et al.* 1974), which more or less compensates for the effectiveness of beta blockade. At a thermoneutral environment there is no need for extra heat production and, as a result, for extra release of catecholamines. It is interesting to note, however that even at the higher temperature repeated *noradrenaline* injections failed to prevent the influence of alprenolol. This may be due to the shorter duration of the action of noradrenaline (*cf.* Fig. ., Harri 1977 b) as compared with that of alprenolol. These results also emphasize that to perform animal experiments in a conventional way at a room temperature is not always the proper way.

The activation of the brown fat in a cold environment is mainly due to the direct effect of the sympathetic nervous system on this tissue (Seydoux 1977). Thus repeated injections of noradrenaline in order to *mimic* the enhanced release of this amine which occurs at cold should lead to changes, similar to those resulting from cold acclimation. The elevated size of this tissue, as observed in this study for 19–21 °C-acclimated rats and earlier by LeBlanc and Villemaire (1970), demonstrate that this is the case. Furthermore, this enlargement was partially antagonized by simultaneous alprenolol treatment.

Unlike at room temperature, N-treatment did not affect the absolute or relative size of the ISBAT at 28–29 °C. The metabolic activity of the tissue was, however greatly increased by N-treatment, as judged from the enzymatic changes and multilocular picture of the tissue. However it is difficult to state whether or not alprenolol antagonized the effects of noradrenaline, because A-treatment alone induced very similar enzymatic changes. At this higher temperature A-treatment alone reduced the size of ISBAT. This effect is unaffected despite simultaneous N-treatment. It is thus tempting to speculate that the influence of alprenolol was stronger than that of noradrenaline when these drugs were given simultaneously.

In the cooling rate test the antagonizing effect of alprenolol against noradrenaline induced improvement in the ability of rats to withstand body cooling in water was very clear. In fact, the rate of body cooling was even more rapid in the rats having received alprenolol injections in addition to noradrenaline than in the rats having received alprenolol injections only. In swimming rats the rate of body cooling depends on the insulative capacity of the body and on the heat production capacity. When the rats are forced to move, this increases the rate of heat loss by convection, and the insulative properties of the body are of less importance. In swimming rats the rate of body temperature loss is even lower than that of a dead body which has no heat production (Dawson *et al.* 1970). Thus it can be speculated that in swimming rats the rate of body cooling is mainly dependent on the heat production capacity of the body and the possible differences in the cooling rate also reflect differences in the heat production capacity. Basing on this test, it can be concluded that alprenolol injections very clearly abolished the effect of N-treatment on the heat production capacity.

Increased non-shivering thermogenesis, *i.e.* calorigenic response to injected noradrenaline has been reported to result from *repeated noradrenaline treatment* (LeBlanc and Pouliot

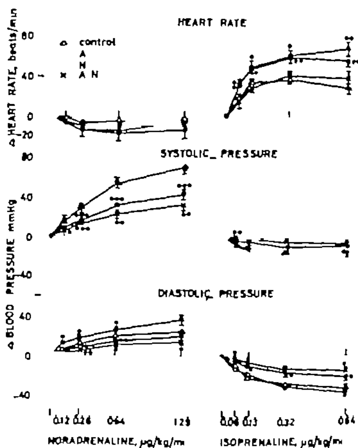


Fig. 3 Effect of noradrenaline and isoprenaline infusions on heart rate, and systolic and diastolic blood pressure i anesthetized rats A = alprenolol treated rats (20 mg/kg daily for 31 39 days), N = noradrenaline treated rats (0.5 mg/kg daily for 31 39 days) and A + N rats i jected with alprenolol together with noradrenaline Each oint represents the mean ± S.E. of 8 to 10 animals. Responses that differ significantly from controls are marked by asterisks p 0 05 p 0 01 and $p < 0.001$ (Student's t-test).

alprenolol injections in spite of the fact that alprenolol injections alone did not affect the heart size.

Despite marked cardiomegaly there was, however no or only minor enzymatic changes in the myocardium of these animal groups It is interesting to note that physical training, although it also results in marked cardiomegaly or metabolic changes in the skeletal muscle, induces only minor changes in the myocardial enzymes (Gollnick and Ianuzzo 1972, Baldwin *et al* 1977 Harri 1977 b)

In the skeletal muscle enzymatic changes were observable only in the series of experiments which were performed at a thermoneutral temperature In these experiments, a decrease in the activity of the aerobic enzymes was evident both in animals injected with alprenolol only and in the rats having received alprenolol injections in addition to noradrenaline. Since a similar decrease was not observed for animals treated with N only and since it has been shown that prolonged stimulation of the organism by a beta adrenergic agonist, isoprenaline (Harri and Valtola 1975) can increase the oxidative metabolism in the skeletal muscle, it is tempting to conclude that this decrease results from diminished sympathetic influence on the beta adrenoreceptors due to prolonged beta blockade This

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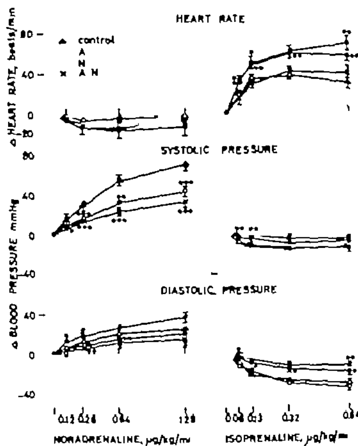


Fig. 3 Effect of noradrenaline and isoprenaline infusions on heart rate, and systolic and diastolic blood pressure in anesthetized rats A alprenolol treated rats (20 mg/kg daily for 31 39 days), N = noradrenaline treated rats (0.5 mg/kg daily for 31 39 days) and A + N = rats injected with alprenolol together with noradrenaline Each of t represents the mean ± S.E. of 8 to 10 animals. Responses that differ significantly from co trols are marked by asterisks p 0.05, p 0.01 and $p < 0.001$ (Student's t-test)

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Despite marked cardiomegaly there was, however no or only minor enzymatic changes in the myocardium of these animal groups. It is interesting to note that physical training, although it also results in marked cardiomegaly or metabolic changes in the skeletal muscle, induces only minor changes in the myocardial enzymes (Gollnick and Ianuzzo 1972, Baldwin *et al* 1977 Harri 1977 b)

In the skeletal muscle, enzymatic changes were observable only in the series of experiments which were performed at a thermoneutral temperature. In these experiments, a decrease in the activity of the aerobic enzymes was evident both in animals injected with alprenolol only and in the rats having received alprenolol injections in addition to noradrenaline. Since a similar decrease was not observed for animals treated with N only and since it has been shown that prolonged stimulation of the organism by a beta adrenergic agonist, isoprenaline (Harri and Valtola 1975), can increase the oxidative metabolism in the skeletal muscle, it is tempting to conclude that this decrease results from diminished sympathetic influence on the beta adrenoreceptors due to prolonged beta blockade. This

is supported also by the results obtained from the series of experiments performed at a room temperature. At this environment the impaired thermoregulatory capacity due to beta blockade, results in a compensatory release of endogenous noradrenaline (cf. Harri 1977 a, b, Irving et al. 1974), which more or less compensates for the effectiveness of beta blockade. At a thermoneutral environment there is no need for extra heat production and, as a result, for extra release of catecholamines. It is interesting to note, however that even at this higher temperature repeated noradrenaline injections failed to prevent the influence of alprenolol. This may be due to the shorter duration of the action of noradrenaline (cf. Fig. 2, Harri 1977 b) as compared with that of alprenolol. These results also emphasize that to perform animal experiments in a conventional way at a room temperature is not always the proper way.

The activation of the brown fat in a cold environment is mainly due to the direct effect of the sympathetic nervous system on this tissue (Seydoux 1977). Thus repeated injections of noradrenaline in order to mimic the enhanced release of this amine which occurs at cold should lead to changes, similar to those resulting from cold acclimation. The elevated [mass] of this tissue, as observed in this study for 19–21°C-acclimated rats and earlier by LeBlanc and Villemaire (1970), demonstrate that this is the case. Furthermore, this enlargement was partially antagonized by simultaneous alprenolol treatment.

Unlike at room temperature, N-treatment did not affect the absolute or relative size of the ISBAT at 28–29°C. The metabolic activity of the tissue was, however greatly increased by N-treatment, as judged from the enzymatic changes and multilocular picture of the tissue. However it is difficult to state whether or not alprenolol antagonized the effects of noradrenaline, because A-treatment alone induced very similar enzymatic changes. At this higher temperature A-treatment alone reduced the size of ISBAT. This [effect] was unaffected despite simultaneous N-treatment. It is thus tempting to speculate that the influence of alprenolol was stronger than that of noradrenaline when these drugs were given simultaneously.

In the cooling rate test the antagonizing effect of alprenolol against noradrenaline induced improvement in the ability of rats to withstand body cooling in water was very clear. In fact, the rate of body cooling was even more rapid in the rats having received alprenolol injections in addition to noradrenaline than in the rats having received alprenolol injections only. In swimming rats the rate of body cooling depends on the insulative capacity of the body and on the heat production capacity. When the rats are forced to swim, this increases the rate of heat loss by convection, and the insulative properties of the body are of less importance. In swimming rats the rate of body temperature loss is even faster than that of a dead body which has no heat production (Dawson et al. 1970). Thus it can be speculated that in swimming rats the rate of body cooling is mainly dependent on the heat production capacity of the body and the possible differences in the cooling rate also reflect differences in the heat production capacity. Basing on this test, it can be concluded that alprenolol injections very clearly abolished the effect of N-treatment on the heat production capacity.

Increased non-shivering thermogenesis, i.e. calorigenic response to injected noradrenaline has been reported to result from repeated noradrenaline treatment (LeBlanc and Poullot

1964 LeBlanc and Villemaire 1970 Hsieh and Wang 1971). In this study the hypertherm response to injected noradrenaline was used as a measure of non-shivering termogenes The results showed that repeated N-injections increased this response both in the r maintained at a room temperature and in the rats treated at a thermoneutral temperatu This increased response was very clearly abolished by simultaneous A injections.

After repeated N injections the rats showed decreased hypertonic response to intr venously infused noradrenaline, while in the rats having received repeated alprenolol i jections noradrenaline caused a greater increment in the diastolic pressure. In this ca however the A + N-rats very closely resembled those of N-treated rats, an indication th A treatment did not abolish the changes following N-treatment. Since the pressor respon to noradrenaline is mainly due to vasoconstriction, which is known to be mediated l the alpha-adrenoreceptors, it was to be expected that beta blockade had no antagonis effect on these changes.

By contrast, isoprenaline is known as a pure beta agonist Therefore, it could be expecte that, if prolonged noradrenaline treatment could result in changes in responsiveness t isoprenaline, these changes should be antagonized by alprenolol injections. The prese results show however that repeated N injections resulted in enhanced tachycardic respo to infused isoprenaline which was also observable in the A treated rats but not in t A + N treated rats. An increased tachycardic response to infused isoprenaline has bee reported to occur after repeated noradrenaline injections (Vallieres *et al.* 1972). Since similar sensitization also results from repeated injections of isoprenaline (LeBlanc et a 1972, Vallieres *et al* 1972) it has been concluded that this kind of supersensitivity resul from prolonged stimulation of beta receptors. The supersensitivity following prolonge A treatment is more difficult to explain. A possible explanation is the intrinsic sympth mimetic activity of alprenolol, which in repeated use could activate the cardiac bet receptors. In this case the supersensitivity should be observable in the A + N-treate animals as well Since this was not the case this problem remains to be clarified b further experiments.

The unchanged response of the blood pressure to isoprenaline in the N-treated rats i not in accordance with the findings of Vallieres *et al* (1972) they found a decreased hypo tonic response to isoprenaline in these animals. In general, the hypotonic and tachycardi responses to isoprenaline were much more greater in their studies than those observed in the present study with comparable doses of the drug and with a comparable experiment technique Thus genetical differences between their rats and the animals used in this stud are possible. As to the changed response to isoprenaline in the present study it seem obvious that A-treatment both alone and in combination with noradrenaline led to a decreased hypotonic response to isoprenaline. If it is assumed that this response results from isoprenaline-induced vasodilatation, which is mediated by a the $beta_2$-receptors (Lands *et al* 1967), unlike $beta_1$-receptor mediated cardiac responses, it can be concluded that prolonged beta blockade leads to subsensitivity of $beta_2$-receptors. This effect persists despite simultaneous stimulation of those receptors by noradrenaline, an indication that in this case, too the influence of beta blocking effect of alprenolol exceeds that of beta stimulating effect of noradrenaline, when these both drugs are given together

However, it should be kept in mind that reflex adjustment of circulation greatly affects the cardiovascular reactions in the whole animal experiments. Therefore, altered responses to sympathomimetic amines are not necessary explained by sensitivity changes of adrenergic receptors. In vitro-experiments are needed to clarify these problems in more details.

An unexplainable phenomenon is the enlargement of the adrenals in the 28–29 C [illegible] animals, because it was as remarkable in the A N-group as in the N-treated group. If this enlargement can be interpreted to be due to the stress of repeated noradrenaline loads, it should be absent in the A + N-group, in which, according to the other results presented, alprenolol abolished the effect of noradrenaline. Since this was not the case, this phenomenon cannot be accounted for by the alprenolol-noradrenaline antagonism principle.

In conclusion, the present results show that, if repeated noradrenaline injections resulted in metabolic changes, these changes were abolished by simultaneous alprenolol injections. In those cases in which noradrenaline injections did not induce metabolic alterations but alprenolol injections did, the metabolic alterations in the animals having received both drugs simultaneously more closely resembled those of the animals injected with alprenolol only. The cardiovascular sensitivity changes resulting from N-treatment, which can be supposed to be mediated by the alpha-adrenoreceptors, were not antagonized by A-treatment, [illegible]hile at least some of the changes mediated by the beta-adrenoreceptors, were. These results demonstrate that prolonged alprenolol treatment can protect the organism against the metabolic changes resulting from repeated noradrenaline loads associated with stress situations.

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Inhibition of compound 48/80-induced intradental sensory nerve activity by disodium cromoglycate and serotonin antagonists

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Abstract

OLGART L. and B. GAZELIUS. *Inhibition of compound 48/80-induced intradental sensory nerve activity by disodium cromoglycate and serotonin antagonists.* Acta physiol. scand. 1978 *104* 415–421

Local application of compound 48/80 induced long lasting nerve activity in intradental sensory nerves in the teeth of cats and dogs. This effect was inhibited by pretreatment with disodium cromoglycate (DSCG) given locally (2×10^{-2} M) or i.a. (20 mg/min). DSCG did not have any effect on the nerve excitability *per se* as judged from the responses to hypertonic NaCl and air blasts applied to exposed dentin before and after administration of DSCG. Local treatment with lysergic acid diethylamide (0.1–1 mg/ml) and methysergide (0.05–0.5 mg/ml) selectively reduced or inhibited the compound 48/80 induced nerve activity. Systemic administration of methysergide (12.5 μg/kg) prevented the excitatory effects of compound 48/80 but was without effect when administrated during a state of established activity. The present findings support the hypothesis that compound 48/80 has an indirect effect on intradental sensory nerves and indicate that vascular reactions take part in intradental sensory nerve excitation.

It was recently demonstrated that compound 48/80 causes increased excitability in the sensory nerves in the feline tooth after local application of the drug to exposed dentin (Olgart 1974). These results raised the question of whether this effect of compound 48/80 was due to a direct action on the nerve fibres or an indirect effect mediated by biogenic substances released from mast cells. The latter alternative implies that the induced nerve activity may be a sign of an acute inflammatory reaction in the pulp. However, little is known about the contribution of such reactions to sensory nerve activation and pain in the tooth. Therefore, in the present investigation we have analysed by pharmacological means the excitatory action of compound 48/80 in teeth of cats and dogs.

The present results strengthen the hypothesis that compound 48/80 induces vascular changes in the pulp and suggest that 5-HT is involved in the resulting sensory nerve activity.

Methods

Adult cats, anesthetized with chloralose-urethane and female dogs anesthetized with pentobarbital were used for the experiments. The trachea was intubated and provision made for recording the femoral blood pressure. The experimental set up was similar to that described by Olgart *et al.* (1974).

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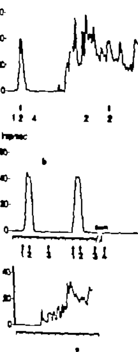


Fig. 1

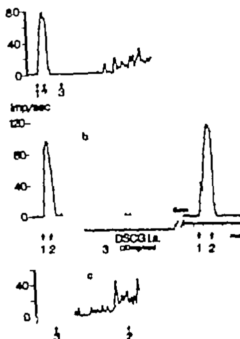


Fig. 2

Fig. 1. Influence of compound 48/80 on sensory nerve activity in the dental pulp. a, expected response to locally applied drugs in the control tooth. b, similar procedures in contralateral tooth after local pretreatment with disodium cromoglycate (DSCG). c, same tooth as in b 30 min. after treatment with DSCG. 1. Sodium chloride, 1.54 M. 2. Sodium chloride, isotonic. 3. DSCG, 2 × 10^{-4} M. 4. Compound 48/80, 1 mg/ml. Impulse frequency represents the running average for 10 s interval and is given as imp/s. Cat, chloralose-urethane.

Fig. 2. The effect of local application of compound 48/80 on intradental sensory nerve activity before and after intraarterially administered disodium cromoglycate (DSCG). a, responses in control tooth prior to DSCG administration. b, contralateral tooth during infusion of DSCG (20 mg/min). Note that stimulation with hypertonic sodium chloride induced similar responses before and after DSCG infusion. c, same tooth as in b 50 min. later. The following solutions were applied locally: 1. Sodium chloride, 0.76 M. 2. Sodium chloride, isotonic. 3. Compound 48/80, 0.1 mg/ml.

Serotonin antagonists

The principal action of DSCG is to stabilize the mast cell membrane (Brodgen *et al.* 1974). The ability of this drug to prevent the excitation induced by compound 48/80, but not the effect of other well-known direct acting stimuli, suggests that compound 48/80 has an indirect effect on the sensory fibres, *e.g.* by releasing some biogenic principle. Since serotonin (5-HT) has been shown to excite intradental neurones (Olgart 1974) application of serotonin antagonists could give further information as to the mechanism of excitation by compound 48/80. Fig. 3 shows the results from one experiment which demonstrates the effect of locally applied LSD on the compound 48/80 induced activity. The compound was washed out from the cavity and replaced by isotonic saline 10 min before LSD was introduced. As can be seen, LSD (0.1 mg/ml) (Fig. 3: 1) caused a partial inhibition of the activity within 30 s.

Two platinum electrodes were inserted into dentinal cavities prepared in the canine tooth. The cavities were filled with isotonic saline and differential recordings were made between the two electrodes. Signals were displayed on a cathode ray tube and fed into equipment for frequency analysis (Edwall and Scott 1971, Haegerstam 1976 a). The potentials obtained using this method have earlier been shown to originate from intradental sensory fibres (Anwill *et al* 1973) and to be associated with pain in man (Edwall and Olgart 1977). As has been previously demonstrated (Haegerstam 1976 c) the present technique enables recordings to be made from a small number of units (generally less than five). No attempts were made in this study to differentiate between impulses of different amplitude The effects of pharmacological agents were quantitatively studied by measurements of changes in total impulse activity For further details concerning the recording technique and analysis see Haegerstam (1976 c).

Compound 48/80 solution (0,1 μl) was introduced into the incisal cavity after removal of the saline solution. Disodium cromoglycat (DSCG) was administered either by application into the incisal cavity or by intraarterial infusion in the posterior auricular artery close to its junction with the external maxillary artery Lysergic acid diethylamide (LSD) and methysergide were administered either locally into the incisal cavity or by infusion. Sodium chloride solution (0.76 M 1.54 M) was used locally in the cavity The latent period of the resulting nerve response is dependent on cavity depth (Olgart 1974) and was used to standardize the preparations when results in pairs of teeth were to be compared. The maximal frequency response to this procedure was also used to test the excitability of the sensory neurons after the various procedures. Aconitine was applied locally into the incisal cavity in some experiments to produce long lasting nerve activity (Haegerstam 1976 b) which served as control The drugs were dissolved in isotonic saline

Results

Disodium cromoglycate

Pairs of lower canine teeth (4 cats and 2 dogs) were used in this series of experiments. In each animal one of the teeth served as control to demonstrate the expected response to hypertonic NaCl and compound 48/80 In the contralateral tooth the cavity was pretreated with DSCG solution (2 10^{-4} M) for 5 min, which was then replaced with isotonic NaCl. Hypertonic NaCl was then applied followed by compound 48/80 solution. It was a consistent finding that DSCG prevented the nervous response to compound 48/80 (1 mg/ml) but not that to 1.54 M NaCl Fig. 1 shows the result obtained in such an experiment As can be seen, a response to compound 48/80 was obtained 30 min later in the tooth previously treated with DSCG This recovery in response was a consistent finding in all experiments. In 4 of the experiments air blasts were included This stimulus was applied to the exposed dentin in the recording cavity before and after application of DSCG Comparison between the two responses showed that the excitability was not influenced by DSCG treatment. When DSCG was given after compound 48/80 (5 procedures in 3 cats) no inhibition or a weak inhibitory effect on the activity was observed.

In a similar series of experiments (3 cats) DSCG was administered by intra-arterial infusion (20 mg/min) before and during the local application of compound 48/80 (100 μg/ml). The results were essentially the same as those reported above. Compound 48/80 failed to induce any nerve activity during the DSCG infusion (9 min) and during the subsequent 10 min (Fig. 2) However the response to 0 76 M NaCl or to air blasts (not shown in the figure) was unchanged when compared with the controls made before the DSCG treatment. When compound 48/80 was applied 90 min after the previous DSCG infusion, it induced nervous activity (Fig. 2 c 3) of similar magnitude to that in the contralateral control tooth (Fig. 2 a 3).

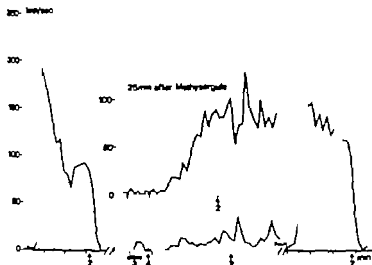


Fig. 5. The effect of compound 48/80 on intradental sensory nerve activity after intravenous administration of methysergide, 12.5 µg/kg (3). The following solutions were applied locally in the recording cavity: 1. Sodium chloride, 0.76 M. 2. Sodium chloride, isotonic. 4. Compound 48/80, 1 mg/ml. Inset shows the response in the same tooth 25 min later.

In 4 expts. methysergide (12.5 µg/kg) was administered i.v. 1 min before the application of compound 48/80. A typical result is shown in Fig. 5. As can be seen, application of compound 48/80 (Fig. 5: 4) after the methysergide administration (Fig. 5: 3) induced activity of low frequency during the following 15 min. The response to 0.76 M NaCl obtained thereafter was similar to the initial control response (Fig. 5: 1). Twentyfive min later a new application of compound 48/80 induced a higher frequency response, which was similar to that obtained in the contralateral control tooth before methysergide administration (not shown in the figure). In contrast to these results, it was found in 3 expts. that methysergide given i.v. after compound 48/80 was unable to inhibit the activity already induced. In 5 control expts. in which aconitine was used to excite the neurones the resulting activity could neither be prevented by a previous injection of methysergide nor be influenced by an injection of methysergide during the established activity.

Tetrodotoxin

The finding that methysergide did not have any effect on the nerve activity when given after compound 48/80 may be explained by a local circulatory arrest in the pulp induced by compound 48/80. An effective distribution of the blocking agent to the pulp would then be prevented. This hypothesis was tested in 3 additional expts. in the following way. Tetrodotoxin (TTX, 3 µg/kg) was given i.v. when intradental nerve activity had been induced by compound 48/80 (0.5 mg/ml) and aconitine (10^{-4} g/ml) in two separate teeth in the same animal. Aconitine is known to act directly on nerves by depolarization of the nerve endings (Wellhöner 1968). TTX has earlier been shown to effectively block aconitine-induced nerve activity as well as activity induced by other means in this preparation (Haegerstam 1976 c).

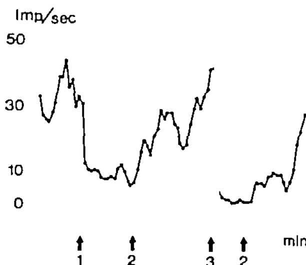


Fig. 3 The effect of lysergic acid diethylamide (LSD) on the sensory unit impulse frequency elicited by compound 48/80 (0.1 mg/ml) Local application in the recording cavity. 1,3 LSD 0.1 and 1 mg/ml respectively 2. Sodium chloride isotonic.

After washing the cavity with isotonic saline, the activity gradually increased during the following 3 min LSD (1 mg/ml) (Fig. 3 3) caused an almost total inhibition of the activity Similar results were obtained with methysergide. In Fig. 4 1 it can be seen that methysergide (0.5 mg/ml) caused a total inhibition of the compound 48/80-induced activity within 1 min after its application. Replacement of the drug by hypertonic NaCl solution (0 76 M) together with methysergide at the same concentration (Fig. 4 2) induced a rapid increase in activity This response indicated that the blocking effect of methysergide of compound 48/80 induced activity was not due to local anesthetic effect. After washing the cavity with isotonic saline (Fig. 4 3) the activity was gradually restored to the initial level. The results in Fig. 4 4 and 4 5 show that two applications of methysergide at lower concentrations (0 1 mg/ml 0 05 mg/ml) markedly reduced the activity These results represent the typical response obtained in 15 procedures in 12 cats. When LSD and methysergide were used in the same preparation methysergide was generally found to be the more potent in inhibiting the compound 48/80 induced activity

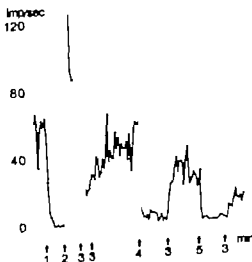


Fig. 4. The effect of methysergide on the sensory unit impulse frequency elicited by compound 48/80. Local application in the recording cavity 1 4 5 Methysergide 0 5 0 1 and 0 05 mg/ml respectively 2. Methysergide 0 5 mg/ml in a solution of sodium chloride, 1.54 M 3 Sodium chloride, isotonic. Note the response to hypertonic sodium chloride with methysergide

In conclusion, the present results strengthen the hypothesis that compound 48/80 activates intradental sensory nerves by an indirect mechanism. It is suggested that compound 48/80 induces vascular changes in the pulp possibly involving 5-HT

The study was supported by grants from the Swedish Medical Research Council (B76-24X-816-11) and Karolinska Institutet.

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In the present experiments, the activity in the aconitine treated tooth was rapidly abolished, while in the compound 48/80 treated tooth the activity was unaffected by the systemically administered dose of TTX. In contrast to these results, local applications of TTX (10^{-4} g/ml) in the teeth were shown to rapidly abolish both types of induced activity. These findings demonstrate that the same local concentration of TTX can block the nerve activity induced by both substances and suggest that compound 48/80 may cause some change in the local circulation under the cavity resulting in an incomplete distribution of the systemically administered TTX to the activated nerves.

Discussion

The inhibition of compound 48/80-induced nerve activity by DSCG in the present expts. is interesting since the drug has a stabilizing effect on the mast cell membrane, but few other pharmacological effects (Cox 1971 Brodgen *et al* 1974 Nuki and Farnoush 1975). Thus mast cell degranultion induced by compound 48/80 in rat peritoneal tissue was reduced by local pretreatment with DSCG in concentrations similar to those used in the present study (Orr *et al* 1971 Marshall 1972). In the present expts, a direct action of DSCG on the sensory nerve excitability may be ruled out, since the preparation showed normal responses to application of hypertonic NaCl and air blasts after DSCG treatment. Our results thus strengthen the hypothesis that nerve excitation induced by compound 48/80 involves an indirect mechanism in which mast cells play a part.

The question arises whether principles released from mast cells have a direct action on the nerves or if the nerve activity is due to some indirect mechanism involving these substances. A direct effect seems less probable since it has been shown that substances which are released or formed as a consequence of cat mast cell degranulation are without excitatory effect in the present preparation (Olgart 1974 Haegerstam and Edwall 1975 Gazelius *et al.* unpublished).

The other alternative is that circulatory changes may be involved. Support for this assumption is found in recent results from studies on the cat tooth (Ahlberg and Edwall 1977 Ahlberg 1978) showing that local application of compound 48/80 reduces pulp blood flow concomitantly with an increase in sensory nerve activity. Further support for a vascular influence is to be found in the results of the present experiments where systemically administered TTX was unable to block nerve activity induced by compound 48/80 but blocked that induced by aconitine. This suggests that compound 48/80 or substances released from mast cells, induces vascular changes that initiate the neural response. Such a mechanism of excitation may be related to a local increase in intrapulpal pressure due to outward filtration of fluid from the vessels (van Hassel 1971) and pressure effects on mechanosensitive sensory receptors (Paintal 1976). A mechanosensitive feature of intradental sensory neurons was suggested by Haegerstam (1976 c).

The present finding that methysergide in a dose range of 10–15 μg/kg was able to block nerve activity induced by compound 48/80 may indicate that 5-HT is involved. 5-HT may take part in the nerve excitation either by exerting direct effect on the nerves (Olgart 1974) or indirectly by its vascular actions.

In conclusion, the present results strengthen the hypothesis that compound 48/80 activates intradental sensory nerves by an indirect mechanism. It is suggested that compound 48/80 induces vascular changes in the pulp possibly involving 5-HT

The study was supported by grants from the Swedish Medical Research Council (B76-24X-816-11) and Karolinska Institutet.

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Acta physiol. scand. 1978. 104. 422–430

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Effects of hyperinsulinemia on lactose secretion and glucose uptake by the goat mammary gland

By

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Abstract

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Lactose secretion by goat mammary glands was studied after intra-arterial infusions of insulin and/or glucose causing rises in plasma insulin concentrations in mammary venous blood of about 3–5 ng/ml. Such levels are sufficient to strongly stimulate insulin sensitive processes when present in the systemic circulation. Two goats with mammary glands in situ and two with one of the glands autotransplanted to the neck were used for the experiments. Increasing the insulin concentration in blood to the mammary gland induced a decrease in lactose yields during 3 h infusion ($p < 0.02$). Simultaneously arterial plasma glucose decreased by 15–20 mg/100 ml to about 50 mg/100 ml. When the systemic hypoglycemic effect of the insulin infusion was offset by a simultaneous intra-venous infusion of glucose no significant change in lactose secretion was observed during 8 hours of hyperinsulinemia. No change in mammary glucose uptake as measured by mammary blood flow mammary arterio-venous glucose differences could be detected. (Average glucose uptake for 2 h before infusion, 30.9 mg/min during 8 h of infusion 31.2 mg/min.) It may be concluded that increased levels of insulin in the blood perfusing the mammary gland did not affect mammary glucose uptake or lactose synthesis as long as blood glucose was maintained at normal levels. The results therefore indicate that the glucose turnover in mammary glands, which in high yielding dairy ruminants represents the main organ of glucose utilization in the body is in fact independant of changes in plasma insulin concentrations.

The glucose uptake of the mammary gland accounts for 60–85 per cent of the glucose utilization in lactating dairy ruminants Two thirds or more of the mammary uptake is converted to lactose (Annison and Linzell 1964 Bickerstaffe, Annison and Linzell 1974). The role played by insulin in this extensive carbohydrate uptake and conversion is, however not fully understood Insulin is essential for the maintenance of secretory activity in mammary tissue in rodents (Walters and MacLean 1968 Martin and Baldwin 1971 *a b*) Lack of insulin for 36–48 h or more reduces milk secretion and enzymatic activity in the mammary gland In goats alloxan diabetes of some weeks duration causes complete and irreversible loss of secretory activity (Nowak and Dzialoszynski 1967). In contrast, the acute effects of insulin deficiency on milk secretion are negligible (Hove 1978)

The effects of increased insulin levels on mammary gland metabolism and secretion rate have been studied both *in vivo* and *in vitro* Bauman *et al* (1973) and Baldwin *et al* (quoted

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in Baldwin and Louis 1975) have shown that insulin, when added to slices of cow mammary gland, has no effect on lipogenesis from acetate or on the pattern of glucose metabolism. An extensive administration of insulin to lactating ruminants reduces both milk volume and lactose production (Gowen and Tobey 1932), but these changes seem to be secondary to the hypoglycemia developed by insulin since they may be prevented by simultaneous intravenous infusions of glucose.

Since marked fluctuations in plasma insulin occur throughout the day (McAtee and Trenkle 1971 Hove and Blom 1973 Hart *et al.* 1975) while milk production is almost constant from hour to hour one might expect that mammary glucose uptake and carbohydrate secretion proceeds independently of the changing concentrations of insulin in blood. To test this two series of insulin infusion experiments were designed using local infusions into a mammary artery In the first series the effects of increased plasma insulin concentrations on lactose secretion were studied during close arterial insulin infusion. In the second series the hypoglycemic conditions resulting from insulin infusion in series one were offset by simultaneous infusions of glucose to maintain normoglycemic conditions. Mammary blood flow glucose extraction and lactose secretion were measured.

Methods

Animals

Adult goats weighing 30–40 kg were used for the study The animals were in mid to late lactation and yielded from 1 to 1.5 kg milk daily The goats were housed indoors in individual pens during the experiments, and were fed hay and pelleted concentrate ration containing 12.5 per cent crude protein *ad lib.* Feed was given twice daily at about 8 a.m. and 4 p.m. and milking was performed in connection with the meals. Feeding and milking were carried out as usual on days of experiment. The goats were allowed to rest undisturbed for [illegible]–1 h before the experiments started.

Surgical preparations

1) Two goats were prepared for local intra-arterial (i.a.) infusions while having the mammary glands [illegible]. All vessels crossing the midline between the two halves of the udder were resected. In addition [illegible] artery was exteriorized (Linzell 1960). To provide access to the arterial circulation of the gland, the external pudic artery was exteriorized in a skin-covered loop. The artery was carefully freed from its accompanying nerves and vessels, side branches were ligated and the vessel exteriorized either close to the midline or on the caudo-lateral surface of the udder The pudic artery loop did not work satisfactorily for the time needed for experimentation, partly due to an increasing tendency to spastic contractions, and partly due to the development of collateral arterial supply To overcome these difficulties mammary autotransplantations were performed.

2) In two additional goats one of the mammary glands was auto-transplanted to the neck according to the method of Linzell (1963) The external pudic artery was anastomosed end to end with the left carotid artery and the external pudic vein side to end with the jugular vein The carotid artery was exteriorized in a skin-covered loop about 5 cm proximal to the anastomosis

Experimental design

Two series of infusions were performed, one of 5 and the other of 10 h duration. The goats were milked after the intravenous injection of 100 mU of oxytocin The first 2 h thereafter were used as a control period. Then followed a 3 or 8 h test period. The first 2 h were used to measure the basal or preinfusion milk production During this period infusions of saline were usually given. Thereafter the effects of insulin and/or glucose infusions were tested for 3 or 8 h

The validity of the present method for studying milk production depends on the assumption that changes in mammary metabolism induced by the infusions can be detected during the infusion period. The measured lactose production may be influenced by lactose synthesized but not milked out during the foregoing

hours. In the perfused mammary gland preparation Hard ick & Linzell (1960) have calculated that the amounts of lactose remaining in a gland after oxytocin-aided emptying is less than one hour's normal lactose production. Changes in lactose secretion occurring during the last 2 h of a 3 h infusion therefore most probably reflect a true change in mammary production of lactose and not remaining lactose produced earlier For the eight hour infusions, this should obviously be the case

In accordance with the results of Linzell (1967 a, b) and Linzell and Peaker (1971) hourly lactose secretion was found to be nearly constant during several 5 h periods of measurements undertaken to establish spontaneous variations in lactose secretion rate A total of 11 periods were studied in 6 goats ith hourly yields ranging from 40–120 mL When the average secretion during the two first hours as set at 100 per cent lactose secretion during the next 3 h was 100±9 103±8 and 103±11 % (mean and S.D.), respectively. As a consequence hourly milking was used in the 3 h infusion experiments. When oxytocin-aided milking was continued f r periods of up to 10 h, Linzell and Peaker (1971) observed increases in milk yield and slight changes in milk composition To minimize these effects of the oxytocin injections, the mammary glands were left undisturbed for the first 6 h of the 8 h insulin infusion.

During a preliminary series of expts. it was found that elevations of plasma insulin concentrations to 2.5–4 ng/ml in the systemic circulation invariably led to hypoglycemia and reduced milk production within 2 3 h Thus, t ensure a proper stimulation of the possibly insulin sensitive processes in the mammary gland i the present study the dose of insul n infused locally to the mammary arteries were adjusted so as to give blood concentration in the gland of at least 3 g insulin per ml.

Series 1 Short term (3 h) insulin infusions. Insulin (porcine Leo®-neutral) diluted in saline was infused at a rate of 2 μg/kg h i to the exteriorized mammary artery on 5 occasions in 4 goats. The infusate was given through a 1 0 mm (o.d) plastic catheter (Venflon®) previously inserted into the artery Insulin concentrations in the desired range were obtained n the mammary vein, but simultaneously the arterial plasma glucose concentrations decreased considerably

Serie 2. Long t m (8 h) infusions of insulin and glucose Insulin was infused locally to the mammary artery (2 μg/kg h) for 8 h. Simultaneously glucose was i fused to the contralateral jugular ein at a rate of approximately 2 mg/kg min to maintain normoglycemia The infusion rate was adjusted according to variations in the concentration of glucose during the expt Two expts. with each of the two goats ith autotransplanted glands were carried out

Milking I travenous injection f 100 mU of synthetic o ytocin was given t evok milk ejection. Five minutes elapsed from o ytoci dm istration to the completion of milking. The goats were milked by hand and the volumes from the two udder hal es were measured separately Aliquots of 5 ml ere taken after thorough mixing, cooled on ice dur g the expt and then stored t −20°C until analysed.

Blood sampling Catheters for blood sampli g (o.d. 1 0 mm) were inserted into the carotid or mammary artery nd mammary venous loops under local anesthesia. In the two goats with mammary glands in situ the external pudic veins were compressed manually during enous sampling to force enough blood to leave the gland through the caudal epigastric (milk) vein where the sampling catheters were located (Linzell 1960). M mmary venous blood from the autotranspla ted gland was sampled from the jugular vein after compression of the vein distal t the anastomosis with th mammary vein. About 2 ml of blood as withdrawn each time After prompt centrifugation n heparinized tubes the plasma was removed and kept on ice until termination of the expt. Plasma was then frozen and kept t 20°C until analysed The catheters were filled with heparinized saline (5 IU/ml) between samplings. Two pairs of arterial and venous blood samples were taken every hour 20 and 50 min after each oxytocin injection.

Mammary blood flow was measured in the goats with autotransplanted glands by means of an electromagnetic flow meter (Nycotron 376). A cuff type probe (5 or 6 mm I.d.) was chronically implanted around the carotid artery 5–8 cm pro imal to the loop some weeks prior to the beginning of the expts. The leads were tunneled under the ski nd taken out on the middle of the neck The probes remained in position and worked well for several months. Zero blood flow was obtained by clamping of the carotid loop distal to the probe. The zero readi gs were repeatedly hecked during the experiments. The flow signal from the probe was calibrated at the start and t the end of each experiment using digital i tegrator (Nycotron 394). 20 ml of blood was withdrawn by syringe from the clamped artery and the integrated flow signal used to relate instrument output to actual flow values. Usually this calibration procedure wa repeated 3 to 5 times at the beginning and end of the expts. The coefficient f variation f the calibration procedure ranged between 4 and 10% with typical values of 6–7%. The flow signals were recorded on a moving chart and average hourly flow rates calculated afterwards.

Plasma glucose insulin and milk lactose were measured as reported earlier (Hove 1978 in press).

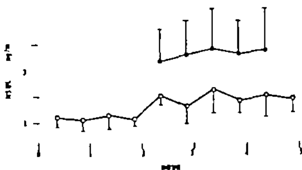


Fig. 1 a.

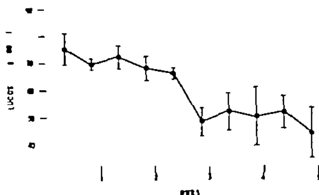


Fig. 1 b.

Fig. 1. (a) Arterial (○) and mammary venous (●) insulin concentrations before and during the intra-arterial infusions of insulin [illegible] series I. (b) Arterial glucose concentrations. Infusion indicated by black bar. Means and S.D. (5 [illegible])

Statistics. Student t-test for paired comparisons was used to evaluate changes in lactose secretion. The [illegible] rates in the preinfusion periods were compared with the secretion during the last two hours of the infusion both in the short and long-term infusion expts. $P < 0.05$ was taken to represent statistical significance.

Results

Series I. Short term insulin infusions. The infusion of insulin into the artery of one of the mammary glands—either *in situ* or autotransplanted—raised the insulin concentration in mammary venous plasma to between 3.5 and 4 ng/ml (Fig. 1 a). The plateau levels obtained in the mammary veins were significantly higher than the insulin concentrations in the systemic circulation ($p < 0.05$).

The systemic arterial insulin concentration, and hence the insulin concentration in the blood perfusing the other gland, rose from 1.25 before the expt. to about 2 ng/ml at the end of the infusion (Fig. 1 a). The infusion of insulin resulted in a decrease of the arterial plasma glucose by 15–20 mg/100 ml to about 50 mg/100 ml (Fig. 1 b). A reduction in lactose secre-

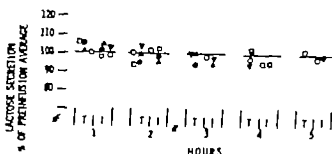


Fig. 2. Lactose secretion before and during the i.a. infusion of insulin in series I. Lactose secretion in per cent of the average secretion for each gland during the two-hour pre-infusion period. T and I: Transplanted gland and the gland remaining *in situ* respectively. In the goat with both glands *in situ* T represents the infused gland. Black bar indicates the period of infusion to T

tion was observed during the last two hours in most glands (Fig. 2). No differences between infused and non-infused glands could be detected. Thus when all observations of individual gland results were combined, an average reduction of lactose yield to 91 per cent of the pre-infusion yield was observed ($p < 0.02$)

Series 2 Long term infusions of insulin and glucose In these experiments plasma insulin concentrations in mammary venous blood reached a plateau level of about 4.5 ng/ml while systemic insulin concentrations increased from about 1 to between 2.5 and 3 ng/ml (Fig. 3 a) The considerable increase in systemic insulin was counter balanced fairly well by the intravenous infusion of glucose, thus avoiding the development of the hypoglycemia observed during the short term infusion (Fig. 3 b)

Lactose secretion remained at a constant level for the complete infusion period. No changes in lactose secretion could be observed between the infused, transplanted glands and the glands remaining *in situ* (Fig. 4)

Mammary glucose uptake was calculated from plasma flow and ateriovenous plasma glucose differences. Since lactose secretion rates and glucose uptakes were practically identical in the two goats during this series of the experiment, actual uptake figures are presented in Fig. 5 Although the figures for glucose uptake differed significantly for some of the one hour periods, averages for pre infusion and infusion periods were practically identical (30.9 as compared to 31 2 mg/min) The lactose secretion from the glands accounted for 80.5 and 82.0 per cent of the average glucose uptake in the pre-infusion and insulin infusion periods respectively Mammary blood flow increased throughout the experiments in both goats 30–60 per cent higher values were observed at the conclusion of the infusions than in the pre-infusion period Concommittantly a similar reduction in a-v differences of glucose occured, thus giving relatively constant glucose uptake figures. Milk yields of about 0 6 ml/min were recorded during this series of experiments, and the average ratios of blood flow to milk secretion were between 520 and 670.

Discussion

The infusion of insulin into the mammary artery gave rise to average mammary vein insulin concentrations between 3 5 and 4 5 ng/ml and systemic insulin concentrations of about 2 ng/ml. The infusion further resulted in a reduction of about 20 mg/100 ml in plasma glucose.

Glucose seems to be a rate limiting substrate for milk synthesis when available in sub-

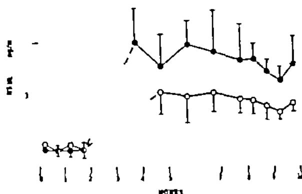


Fig. 3 a.

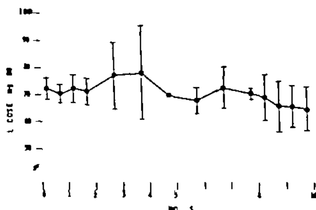


Fig. 3 b.

Fig. 3 (a) Arterial (O) and mammary venous (●) insulin concentrations before and during the 8 h infusion of insulin ( ) and glucose ( ). (b) Arterial glucose concentrations. Infusion indicated by black bar. Means and S.D. (2 expts. in each of 2 goats).

optimal amounts (Hardwick *et al.* 1961 Kronfeld *et al.* 1963, Rook and Hopwood 1970). The reduction in mammary lactose secretion observed during the last part of the insulin infusion most probably therefore resulted from the reduced plasma glucose levels. In fact, the effects of the hypoglycemia which developed during the first hour of the infusion were clearly evident on the lactose secretion after the second hour This rapid decrease in lactose secretion emphasizes the need for a continuous supply of glucose to maintain a constant rate of mammary lactose synthesis.

When in the combined infusion experiments the systemic effects of insulin on plasma glucose were offset by i. glucose, no change in mammary glucose uptake or lactose secretion could be observed. These findings should be compared to the results of a series of expts. where alloxan diabetic lactating goats were controlled by insulin infusions so as to maintain normal blood glucose concentrations (Hove 1978). When insulin treatment was discontinued plasma insulin declined to very low levels and prominent signs of insulin

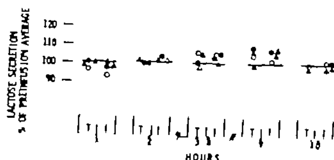


Fig. 4 Lactose secretion before and during the 8 h infusion of insulin (i.a.) and glucose (i.v.). Text as in Fig. 2.

deficiency developed in the systemic circulation in the course of 1–? h. Mammary lactose secretion continued in an unchanged manner for 4 h after withdrawal of the insulin treatment, however. It therefore appears that the mammary secretion of lactose seems to be unaffected by short term (4–8 h) changes in the concentration of plasma insulin. Alternatively the possibility excists that the hyperinsulinism per se could induce endocrine changes which would interfere with or even mask the effects of insulin. Little is known, however, about the hour to hour regulation of mammary metabolism during established lactation. Increased plasma levels of catecholamines might interfere with the mammary substrate supply by inducing vasoconstriction. In the present expts. constant or increasing mammary blood flows during the infusion period seem to exclude this possibility. Although changes in plasma levels of prolactin probably occur as a result of the experimental procedures (milking; Bryant *et al.* 1970) most evidence seems to indicate that changes in plasma prolactin is without effect on milk yields during an established lactation (Cowie 1971, Karg *et al.* 1972, Hart 1973). Hart and Flux (1973) have demonstrated that increases in the plasma concentration of growth hormone may occur in the goat after milking. The possible role of this response in regulating mammary metabolism remains unclear, however.

The findings of blood to milk flow ratios between 500 and 700 is in accordance with figures obtained by thermodilution flow measurements in cow and goat (Linzell 1960, Bickerstaffe *et al.* 1974). The amount of glucose accounted for by lactose secretion (80–82 per cent) is within the upper range of what has been reported earlier (Annison and Linzell 1964). Glucose oxidation or output as milk fat glycerol were not measured in the present study, but it seems probable that these other processes utilizing glucose were proceeding

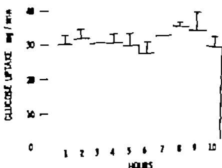


Fig. 5 Mammary glucose uptake in the transplanted gland before and during the 8 h infusion of insulin (i.a.) and glucose (i.v.). Infusion indicated by black bar. Means and S.D. of 2 expts. in each of 2 goats.

at about the same rate in the pre-infusion and infusion periods, when the lack of effects of hyperinsulinemia on glucose uptake and lactose secretion is recalled.

Since the changes in plasma insulin concentration observed in connection with feeding usually is of rather short duration (1 4–6 h) and of smaller magnitude than those imposed by the present infusions, the conclusion can be drawn that the changes in plasma insulin which occurs during ordinary feeding conditions probably do not participate directly in the regulation of mammary carbohydrate uptake and secretion. Numerous studies have stressed the importance of the mammary gland in the utilization of available glucose. In the goat Annison and Linzell (1964) have demonstrated that 60–85 per cent of the glucose entry rate is used by the mammary gland, and comparable figures ha e been obtained by others in the lactating cow (Bickerstaffe *et al* 1974 Patterson and Linzell 1974). The implications of the present findings would be that the major outflow of glucose during lactation in ruminants is independent of short term fluctuations in plasma insulin concentrations.

The skilled technical assistance of Mr Per Morn is acknowledged. The study as supported by the Norwegian Agricultural Research Council.

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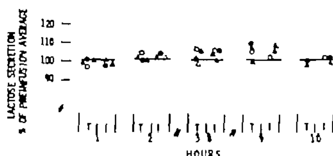
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F g. 4 Lactose secretion before and dur the 8 h infusion of insulin (i a) a glucose (l.). Text as in Fig. 2.

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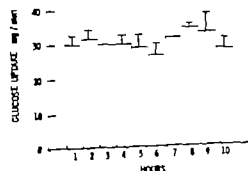


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Acta physiol. scand. 1978. 104. 431–442

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The distribution and secretion of kallikrein in some exocrine organs of the rat

By

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Abstract

ØRSTAVIK, T. B. *The distribution and secretion of kallikrein in some exocrine organs of the rat* Acta physiol. scand. 1978. *104* 431–442.

Glandular kallikrein was quantitated in the submandibular, sublingual, and parotid glands and in the kidney. No kallikrein was detected in the exorbital lacrimal glands and tears. The highest kallikrein concentrations (EU/ml) were in all major salivary gland secretions seen after α-adrenergic stimulation, less after β-adrenergic and least after parasympathetic stimulation. When taking into account the large variations in salivary flow rate, α-adrenergic stimulation was in the parotid and particularly in the submandibular gland found to result in the highest kallikrein secretory activity measured by the kallikrein secretory rate (EU/min). This shows that the kallikrein-rich granular tubular cells are selectively activated through α-adrenergic sympathetic receptors. The differences observed in the parotid saliva were small and not always statistically significant. However, when cervical nerve stimulation was superimposed upon parasympathetic stimulation, kallikrein secretory rate as well as kallikrein concentration increased. The large individual variations in salivary gland kallikrein content and secretion and the rather small differences observed in kallikrein secretory rate after nervous stimulation of the parotid and sublingual glands, may indicate that the kallikrein-containing striated ducts are also influenced by factors other than the secretory nerves. The kallikrein concentration and secretory rate in urine was studied. A strong positive correlation between kallikrein secretory rate and fluid volume was found in urine but not in saliva.

Glandular kallikreins are serine proteinases (E.C.3.4.21.8) able to release kinins from kininogen. In mammals they are found in the kidney and in exocrine glands as well as in the secretions of these organs. In the major salivary glands of the rat kallikrein is confined to the duct system (Ørstavik *et al.* 1975, Brandtzaeg *et al.* 1976, Ørstavik *et al.* 1977, Ørstavik 1978). Stimulation of glandular kallikrein secretion has previously been investigated in the rat submandibular gland (Ørstavik and Gautvik 1977). However, this gland differs from the other major salivary glands by the presence of granular tubules, a structure particularly rich in kallikrein (Ørstavik *et al.* 1977). Therefore, interpretations on kallikrein function in general cannot be based on the stimulatory pattern of this gland alone. This report describes an extended study of the kallikrein secretion in the major salivary glands of the rat. Furthermore, a comparison of enzyme content, cellular localization, and kallikrein secretion is made among various kallikrein-containing organs.

Materials and Methods

Animals. Female Sprague Dawley rats were used (14–16 weeks, 250–300 g b.wt.). They were given tap water and food ad lib. The animals were anaesthetized with intraperitoneal injections of pentobarbital (70 mg/kg b.wt.). Animals used for collection of saliva were tracheotomized and maintained spontaneous respiration during the experimental period. Those used for collection of urine were kept in metabolic cages and were fed on ground pellets and water *ad lib.*

Gland homogenates. The parotid, submandibular and sublingual salivary glands, and the exorbital lacrimal glands were dissected out and frozen at −70°C until used within about 4 weeks. After thawing, fat and capsular material were removed, the glands were cut into pieces, suspended in PBS (0.01 M Na-phosphate buffer pH 7.4 and 0.15 M NaCl), 1:10 wt./vol., and homogenized in a Potter Elvehjem homogenizer (40 strokes, 4°C). After centrifugation (20 000 × g, 20 min, 4°C) the supernatant fluid was collected and frozen (−20°C) for subsequent measurements. The sediment of the submandibular gland homogenate was re-homogenized twice in the same volume of buffer and the 3 supernatant fluids were combined. Due to the small size of the sublingual gland 4 or 6 glands were pooled to give one homogenate, and 4 different homogenates were made. With the other organs one homogenate was produced for each gland.

Collection of saliva. The main excretory ducts of the parotid, sublingual and submandibular glands were cannulated with a polyvinyl ethylene tubing (PP 10 Portex), which was pulled out when used for the two first. The parotid duct was dissected free from the masseteric muscle and cannulated distal to the point where it leaves the marginal mandibular branch of the facial nerve. The ducts of the submandibular and sublingual glands were exposed by removal of the mylohyoid and digastric muscles. The two ducts were carefully separated under a dissecting microscope. The submandibular duct was medially situated to the sublingual duct and showed a larger lumen. The latter was also recognized by its content of mucous and thread-drawing saliva. Retrograde injection of tryptophane blue solution was occasionally performed after the fluid collection to control the cannulation. In each animal the parotid glands were cannulated bilaterally whereas for the submandibular and sublingual glands only one of the two was cannulated on the same side. Saliva was collected during the gland activation period into tarated tubes, and the volume of saliva was estimated by weight (1 μl = 1 mg).

Stimulation of salivary gland secretion. Parasympathetic stimulation was obtained by (a) injections of pilocarpine (0.4 ml, 4 g/l) into the femoral vein, (b) injection of acetylcholine (0.1 ml, 0.1 g/l) into the femoral vein, (c) electrical stimulation of the auriculotemporal nerve activating the parotid gland or of the nerve plexus surrounding the ducts of the submandibular and sublingual glands (7.5 V, 2 ms duration and 9.5 Hz). Sympathetic stimulation was performed by (a) intermittent electrical stimulation of the sympathetic cervical nerve (7.5 V, 2 ms duration and 9.5 Hz), (b) injection of norepinephrine (0.06 ml, 1 g/l) into the femoral vein or (c) injection of isoproterenol (0.15 ml, 1 g/l) also into the femoral vein. Saliva was usually collected for 10–15 min or after norepinephrine administration until the rat died from pulmonary edema.

Collection of urine. The rats were kept in metabolic cages for 2 days before the collection started. Urine was then accumulated during periods of 24 h for 15 days. Toluene was added to the collecting glass to prevent bacterial growth. The urine was centrifuged (10 000 × g, 20 min, 4°C), and frozen (−20°C).

Collection of tears. Tears were collected from the corneal surface of one rat after electrical stimulation of the temporal nerve for about 2 min (7.5 V, 2 ms duration, 9.5 Hz). Attempts to cannulate the duct of the exorbital lacrimal gland were unsuccessful.

Kallikrein measurement. Kallikrein was quantified as described previously (Ørstavik *et al.* 1977) by its antigenicity in a single radial immunodiffusion system and by its Bz-Arg-OEt-esterase activity. For the exorbital lacrimal gland homogenates and the tears 10–15 μl were incubated for up to 4 h at 37°C with the Bz-Arg-OEt-substrate. A small volume of buffer was added to the sublingual gland saliva before it was frozen to prevent desiccation, and therefore this secretion also had to be incubated for 4 h. All other samples were incubated from 5 to 60 min in addition providing a measurable breakdown rate of the substrate.

The rabbit antiserum to submandibular gland kallikrein used in the SRID-measurements contained 32 precipitating units when tested by double diffusion against standard submandibular gland homogenate (0.19 g kallikrein and 2.1 g protein/l).

Statistical analyses. The results are given as mean values ± S.D. Differences between experimental groups were examined by the non-paired, two-tailed Wilcoxon's test for non-parametric data (van Elteren 1960).

Drugs. Pentobarbital (Nembutal sodium®, Abbott Laboratories, London), pilocarpine (NAF Laboratories A/S, Oslo, Norway), acetylcholine chloride (F. Hoffmann-La Roche & Co. A.G., Basel), norepinephrine (Nor-adrenalin®, Norsk Astra A/S, Oslo, Norway), isoproterenol (Isoprenalini sulphas®, Norwegian Medical Depot, Oslo, Norway).

TABLE I. Quantification of kallikrein antigen and Bz-Arg-OEt-esterase activity in homogenates of rat exocrine glands.

	Number of glands	Kallikrein antigen (μg/g)[a]	Bz-Arg-OEt-esterase activity (EU/gland)	(EU/g)[a]	% esterase activity representing kallikrein[b]
Submandibular gland	14	11 950 ± 2 253	2 101 ± 682[c]	9 578 ± 384	78 ± 16[c]
Sublingual gland	4	126 ± 5	3.9 ± 0.5	117 ± 28	70 ± 9
Parotid gland	5	Below detection limit	0.8 ± 0.5	3.9 ± 2.5	
Exorbital lacr. gl.	6	Absent or below detection limit			
Pancreas (not act.)[d]	8	132 ± 51	0	0	

[a] Concentration on the basis of glandular wet weight.
[b] The percentage of total Bz-Arg-OEt-esterase activity representing kallikrein was calculated individually for each gland using the specific esterase activity of purified submandibular gland kallikrein, i.e. 599 EU/mg (Brandtzaeg et al. 1976).
[c] Data obtained from Orstavik and Gautvik (1977).
[d] The values were obtained from Orstavik and Glenner (1978). In the pancreas glandular kallikrein is found as a pro-enzyme. Therefore, no kallikrein esterase activity was detected in non-activated gland homogenates.

Results

Quantification of kallikrein in gland homogenates. The concentration of kallikrein in the various exocrine organs of the rat is given in Table I. In the parotid gland the kallikrein concentration was too low to be detected by the SRID. No esterase activity was found in the non-activated pancreas homogenates where kallikrein is found as a pro-enzyme (Orstavik and Glenner 1978). In the exorbital lacrimal gland neither kallikrein antigen nor Bz-Arg-OEt esterase activity was detected.

Salivary flow rate. Resting saliva was only seen in the sublingual gland and then only in very minute quantities. Parasympathetic stimulation induced a rich flow of saliva from all of the major salivary glands (Table II). The flow rate from the submandibular gland decreased during the stimulation period, as did the pilocarpine induced secretion from the parotid gland. Salivary secretion following stimulation of the auricular temporal nerve most often showed a temporary increase during the stimulation period. Sympathetic stimulation also produced saliva from all three glands although with a lower secretory rate than after parasympathetically stimulation (Table II), and with a decrease in flow rate during the stimulation period. In the sublingual gland salivary flow rate induced by sympathetic stimulation was very low (Table II).

Stimulation of salivary kallikrein secretion. The concentration of kallikrein in saliva and its secretory rate from the submandibular, sublingual, and parotid glands are given in Table III. The kallikrein concentration of the parasympathetically induced saliva was for all three glands significantly ($P < 0.01$) lower than that produced by the various adrenergic stimulation procedures.

In the submandibular gland kallikrein secretion was strikingly released by α-adrenergic stimulation (norepinephrine). Also β-adrenergic stimulation (isoproterenol) resulted in some kallikrein secretion giving a higher salivary kallikrein concentration although much lower

Materials and Methods

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Collection of saliva. The main excretory ducts of the parotid, sublingual and submandibular glands were cannulated with a polyvinyl ethylene tubing (PP 10, Portex), which was pulled out when used for the two first. The parotid duct was dissected free from the masseteric muscle and cannulated distal to the point where it leaves the marginal mandibular branch of the facial nerve. The ducts of the submandibular and sublingual glands were exposed by removal of the mylohyoid and digastric muscles. The two ducts were carefully separated under a dissecting microscope. The submandibular duct was medially situated to the sublingual duct and showed a larger lumen. The latter was also recognized by its content of mucous and thread-drawing saliva. Retrograde injection of a tryptophane blue solution was occasionally performed after the fluid collection to control the cannulation. In each animal the parotid glands were cannulated bilaterally whereas for the submandibular and sublingual glands only one of the two was cannulated on the same side. Saliva was collected during the gland activation period into tarated tubes, and the volume of saliva was estimated by weight (1 μl = 1 mg).

Stimulation of salivary gland secretion. Parasympathetic stimulation was obtained by (a) injections of pilocarpine (0.4 ml, 4 g/l) into the femoral vein, (b) injection of acetylcholine (0.1 ml, 0.1 g/l) into the femoral vein, (c) electrical stimulation of the auriculotemporal nerve activating the parotid gland or of the nerve plexus surrounding the ducts of the submandibular and sublingual glands (7.5 V, 2 ms duration and 9.5 Hz). Sympathetic stimulation was performed by (a) intermittent electrical stimulation of the sympathetic cervical nerve (7.5 V, 2 ms duration and 9.5 Hz), (b) injection of norepinephrine (0.06 ml, 1 g/l) into the femoral vein, or (c) injection of isoproterenol (0.15 ml, 1 g/l) also into the femoral vein. Saliva was usually collected for 10–15 min or after norepinephrine administration until the rat died from pulmonary edema.

Collection of urine. The rats were kept in metabolic cages for 2 days before the collection started. Urine was then accumulated during periods of 24 h for 15 days. Toluene was added to the collecting glass to prevent bacterial growth. The urine was centrifuged (10 000 $\times$ g, 20 min, 4°C), and frozen (−20°C).

Collection of tears. Tears were collected from the corneal surface of one rat after electrical stimulation of the temporal nerve for about 2 min (7.5 V, 2 ms duration, 9.5 Hz). Attempts to cannulate the duct of the exorbital lacrimal gland were unsuccessful.

Kallikrein measurements. Kallikrein was quantified as described previously (Ørstavik *et al.* 1977) by its antigenicity in a single radial immunodiffusion system and by its Bz-Arg-OEt-esterase activity. For the exorbital lacrimal gland homogenates and the tears 10–15 μl were incubated for up to 4 h at 37°C with the Bz-Arg-OEt-substrate. A small volume of buffer was added to the sublingual gland saliva before it was frozen to prevent desiccation, and therefore this secretion also had to be incubated for 4 h. All other samples were incubated from 5 to 60 min in a dilution providing a measurable breakdown rate of the substrate.

The rabbit antiserum to submandibular gland kallikrein used in the SRID-measurements contained 32 precipitating units when tested by double diffusion against a standard submandibular gland homogenate (0.19 g kallikrein and 2.1 g protein/l).

Statistical analyses. The results are given as mean values ± S.D. Differences between experimental groups were examined by the non-paired, two-tailed Wilcoxon's test for non-parametric data (via Elteren 1960).

Drugs. Pentobarbital (Nembutal sodium® Abbott Laboratories, London), pilocarpine (NAF-Laboratories A/S, Oslo, Norway), acetylcholine chloride (F. Hoffmann-La Roche & Co. A.G., Basel), norepinephrine (Nor-adrenalin® Norsk Astra A/S, Oslo, Norway), isoproterenol (Isoprenalini sulphas® Norwegian Medical Depot, Oslo, Norway).

TABLE I Quantification of kallikrein antigen and Bz-Arg-OEt-esterase activity in homogenates of rat exocrine glands.

	Number of glands	Kallikrein antigen (ng/g)[a]	Bz-Arg-OEt-esterase activity (EU/gland)	Bz-Arg-OEt-esterase activity (EU/g)[a]	esterase activity representing kallikrein
Submandibular gland	14	11 950 ± 2 253	2 101 ± 682[c]	9 578 ± 2 384	78 ± 16[b]
Sublingual gland	4	126 ± 5	3 9 ± 0.3	117 ± 28	70 _ 9
Parotid gland	5	Below detection limit	0.8 ± 0 5	3.9 ± 2.5	
Exorbital lacr gl	6	Absent or below detection limit			
Pancreas (not act.)[d]	8	132 ± 51	0	0	

Concentration on the basis of glandular wet weight.
The percentage of total Bz-Arg-OEt-esterase activity representing kallikrein as calculated individually for each gland using the specific esterase activity of purified submandibular gland kallikrein, [illegible] 599 EU/mg (Brandtzaeg et al 1976)
Data obtained from Ørstavik and Gautvik (1977)
The values were obtained from Ørstavik and Glenner (1978). In the pancreas glandular kallikrein is found as pro-enzyme. Therefore, no kallikrein esterase activity was detected in non-activated gland homogenates.

Results

Quantification of kallikrein in gland homogenates. The concentration of kallikrein in the various exocrine organs of the rat is given in Table I. In the parotid gland the kallikrein concentration was too low to be detected by the SRID. No esterase activity was found in the non-activated pancreas homogenates where kallikrein is found as a pro-enzyme (Ørstavik and Glenner 1978). In the exorbital lacrimal gland neither kallikrein antigen nor Bz-Arg-OEt esterase activity was detected.

Salivary flow rate. Resting saliva was only seen in the sublingual gland and then only in very minute quantities. Parasympathetic stimulation induced a rich flow of saliva from all of the major salivary glands (Table II). The flow rate from the submandibular gland decreased during the stimulation period, as did the pilocarpine induced secretion from the parotid gland. Salivary secretion following stimulation of the auricular temporal nerve most often showed a temporary increase during the stimulation period. Sympathetic stimulation also produced saliva from all three glands although with a lower secretory rate than after parasympathetically stimulation (Table II), and with a decrease in flow rate during the stimulation period. In the sublingual gland salivary flow rate induced by sympathetic stimulation was very low (Table II).

Stimulation of salivary kallikrein secretion. The concentration of kallikrein in saliva and its secretory rate from the submandibular, sublingual, and parotid glands are given in Table III. The kallikrein concentration of the parasympathetically induced saliva was for all three glands significantly ($P < 0.01$) lower than that produced by the various adrenergic stimulation procedures.

In the *submandibular* gland kallikrein secretion was strikingly released by α-adrenergic stimulation (norepinephrine). Also, β-adrenergic stimulation (isoproterenol) resulted in some kallikrein secretion giving a higher salivary kallikrein concentration although much lower

TABLE II Salivary flow rate from the submandibular, sublingual, and parotid glands following parasympathetic and sympathetic stimulation.

Stimulation	Submandibular gland $(0.216\pm0.023\ g)^a$				Sublingual gland $(0.037\pm0.004\ g)^a$				Parotid gland $(0.214\pm0.046\ g)^a$			
	µl/min	µl/min/g.w.w.[b]	n	min	µl/min	µl/min/g.w.w.	n[b]	min[c]	µl/min	µl/min/g.w.w.[b]	n	min[c]
Parasympathetic												
Electrical	17.0 ± 9.6	78.7	6	10–30	1.1 ± 0.7	29.7	5	10–14	8.3 ± 3.2	38.8	5	10
Pilocarpine	7.1 ± 2.6	32.9	5	20–30	3.6 ± 0.7	97.3	7	15	7.8 ± 3.0	36.4	15	6–28
Acetylcholine	8.7	40.3	2	20	0.3	8.1	1	2	2.8 ± 2.8	13.1	4	2 3
Sympathetic												
Cervical nerve	4.4 ± 1.4	20.3	13	3–15	0.07 (0.03–0.12)	1.9	3	6–10	1.8 ± 1.3	8.4	15	7–15
Norepinephrine	1.9 ± 0.9	8.8	5	3–15	0.03 (0.01–0.04)	0.8	3	5–15	1.3 ± 1.1	6.1	7	3–13
α-adrenergic	2.6 ± 2.0	12.0	7	5–20								
Isoproterenol	0.8 ± 0.6	3.7	6	20–40	0.05 ± 0.04	1.4	4	15–16	1.4 ± 0.6	6.5	8	15–16
β-adrenergic	1.5 ± 0.5	6.9	5	15–30								

[a] Wet weight of the glands.
[b] Salivary flow rate related to the wet weight of the glands.
[c] The length of the stimulation period.
α-adrenergic = cervical nerve stimulation with the addition of a β-blocker (propranolol).
β-adrenergic = cervical nerve stimulation with the addition of an α-blocker (phentolamine).
n = number of glands; when only three glands were used the range of results is given in parentheses below the mean.

than after α-adrenergic stimulation. Parasympathetic saliva contained comparably little kallikrein although of the same concentration order as parotid saliva. The high α-adrenergic release of kallikrein was a true adrenergic induced kallikrein secretory response not due to the lower salivary flow rate following norepinephrine stimulation since also the kallikrein secretory rate, EU/min, was very high (Table III). The kallikrein secretory rate following β-adrenergic (isoproterenol) stimulation, on the other hand, did not differ much from that seen after parasympathetic stimulation. Electrical stimulation along the submandibular gland duct resulted in a saliva with a somewhat higher kallikrein concentration ($P<0.10$) and a higher kallikrein secretory rate ($P<0.01$) than pilocarpine stimulation.

In the *parotid* gland, like in the submandibular gland, sympathetic stimulation resulted in a higher kallikrein concentration than did parasympathetic stimulation ($P<0.01$). The acetylcholine induced parotid saliva was an exception with a remarkably high kallikrein concentration (Table III). The difference between parasympathetically and sympathetically stimulated saliva was not significantly valid when taking into account the differences in salivary flow rate, although the same tendency was observed (Table III). However, when in the parotid gland sympathetic stimulation was superimposed on parasympathetic stimulation, kallikrein secretory rate as well as kallikrein concentration increased in the two animals investigated (Fig. 1a and b). α-adrenergically stimulated saliva had a significantly higher kallikrein concentration ($P<0.05$) and secretory rate ($P<0.01$) than did β-adrenergic parotid

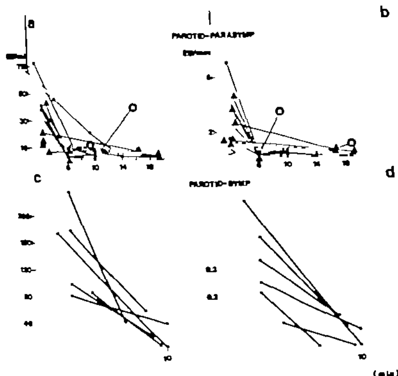


Fig. 1. Salivary kallikrein concentration (a and c) and secretory rate (b and d) during the period of parasympathetic (auriculotemporal nerve ● and pilocarpine ▲) and sympathetic (cervical nerve) stimulation of the parotid gland. Stimulation of the sympathetic cervical chain was superimposed on parasympathomimetic stimulation ○ and resulted in increased kallikrein concentration (a) as well as kallikrein secretory rate (b).

saliva. For the *sublingual* gland saliva similar pattern in kallikrein concentration as that for the parotid gland was seen. However in this gland no differences could be observed in the kallikrein secretory rates.

Individual variations in kallikrein secretion. The kallikrein concentration and secretory rate varied greatly from animal to animal. Standard deviation of mean kallikrein concentration was for instance for pilocarpine stimulated parotid saliva 33.2% and the kallikrein secretory rate varied by 46.4%. However when both glands in a pair were cannulated and exposed to the same stimulation, the saliva of the two glands differed less than among various animals (Table IV). The difference between paired glands is an indication on methodological errors in the test system.

Variations in kallikrein secretion during the stimulation period. The concentration and secretion of kallikrein in *parotid* saliva following parasympathetic stimulation (electrical stimulation of the auriculotemporal nerve or pilocarpine) and electrical stimulation of the sympathetic cervical nerve was found to be much higher during the first 2 3 min than during the following minutes (Fig. 1). During this initial period a large part of the parotid gland kallikrein was secreted, i.e. 0.71 ±0.44 EU for auriculotemporal nerve stimulation (−5) and

TABLE III. Concentration and secretory rate of kallikrein in parasympathetically and sympathetically induced saliva from the submandibular, the sublingual, and the parotid glands, and in urine and tears. The *standard deviation for the sympathetic submandibular saliva* was very high due to the large variations. The range of the kallikrein concentration and secretory rate is therefore given below in parentheses. n = number of glands investigated. When n = 3 the range is given in parentheses below the mean. n* 15 measurements were made in each of the 14 rats. The figure in the table represents the mean of the mean of each rat, thus the standard deviation indicates the *biological variation between different animals*. The day to day variation was found to average 14 ± 4 % and 32 ± 6 % for the kallikrein concentration and secretory rate respectively

	Submandibular gland				Sublingual gland				Parotid gland			
	Kallikrein concentration EU/ml	Kallikrein secretory rate EU/min	n	Stim. period min	Kallikrein concentration EU/ml	Kallikrein secretory rate EU/min 10^{-4}	n	Stim. period min	Kallikrein concentration EU/ml	Kallikrein secretory rate EU/min	n	Stim. period min
Parasympathetic												
Acetylcholine	137.4	1.20	1	20					58.8 (49.5–64.9)	0.095 (0.025–0.187)	3	6–20
Electrical	*155.6 ± 130.9*	*1.76 ± 1.07*[a]	*6*	*10–30*	*1.96 ± 0.77*	*19 ± 9*	*5*	*9–14*	*18.3 ± 11.4*	*0.100 ± 0.047*	*4*	*10*
Pilocarpine	42.3 ± 20.3	0.31 ± 0.16	5	20–30	0.72 ± 0.37	24 ± 9	7	15	8.9 ± 3.4	0.073 ± 0.036	15	6–19
Sympathetic												
Cervical nerve	113 286 ± 156 720 (7 539–560 367)	1 157 ± 2 706 (38–9 973)	13	3–20	22.5 ± 17.0	11 ± 7	4	6–10	85.9 ± 81.9	0.098 ± 0.057	15	8–15
Norepinephrine	88 290 ± 111 522 (13 898–274 500)	201 ± 232 (15–494)	5	3–10	71.8	14	2	5–15	119.6 ± 70.6	0.121 ± 0.048	7	4–18
Isoproterenol	1 984 ± 1 669) (725–4 425	1.3 ± 1.3[a] (0.04–3.5)	6	20–40	22.1 (8.7–42.3)	16 (2.8–42.3)	3	15	42.9 ± 19.1	0.052 ± 0.017	8	15–16
Unstimulated urine:	0.47 ± 0.05 EU/ml (± 10.6 %)	7.2 ± 2.9 EU/24 hrs n* = 14 (± 40.3 %)										

Parasympathetically stimulated tears, no esterase activity

[a] Results published in Ørstavik and Gautvik (1977).

TABLE IV The enzyme concentration and secretory rate in saliva from the major salivary glands following various gland activation procedures. The table shows the difference between paired glands (L and R) and between different animals.

			EU/ml	Deviation from mean %	EU/ml	Deviation from mean %
Submandibular	Pilocarpine	L	34.5	10.3	0.19	22.7
		R	42.4		0.24	
	Acetylcholine	L	147.4	7.2	1.31	10.5
		R	127.3		1.08	
Parotid	Pilocarpine	L	6	3.4	0.043	0
		R	6.7		0.043	
	Pilocarpine	L	14.3	2.9	0.110	10.5
		R	15.2		0.089	
	Pilocarpine	L	8.3	6.7	0.051	24.2
		R	9.5		0.084	
	Pilocarpine	L	5.0	11.5	0.044	19.[illegible]
		R	6.3		0.065	
	Isoproterenol	L	44.6	11.3	0.053	14.5
		R	36.2		0.071	
	Isoproterenol	L	70.6	7.8	0.054	22.7
		R	60.4		0.034	
	Isoproterenol	L	15.0	52.5	0.024	47.0
		R	48.2		0.067	
	Isoproterenol	L	49.5	44.0	0.067	16.5
		R	19.0		0.048	
	Acetylcholine	L	49.5	13.5	0.025	48.9
		R	64.9		0.073	
	Auriculotemporal	L	17.2	17.5	0.083	33.7
		R	24.5		0.168	
Sublingual	Pilocarpine	L	0.57	10.9	0.0022	11.1
		R	0.71		0.0029	
	Pilocarpine	L	1.25	0.4	0.0033	4.9
		R	1.24		0.0037	
	Pilocarpine	L	0.44	8.5	0.0020	42.3
		R	0.27		0.0008	
Mean of deviation from mean				13.9 ±14.7		21.9 ±15.0

0.91 ± 0.32 EU for cervical nerve stimulation (n = 5). The parotid gland contained 0.82 ± 0.54 EU (n = 5).

In the *submandibular* gland parasympathetically induced saliva showed a time-dependent decrease in kallikrein concentration and secretion (Fig. 2a and b) which was comparable to that seen in the parotid gland. Following sympathetic stimulation (Fig. 2b and c) 4 out of 5 animals showed a decrease in kallikrein concentration and secretory rate during the stimulation period. In these rats a considerable amount of total gland kallikrein was secreted during the initial stimulation period. In one animal the kallikrein concentration remained unaltered. However, this particular gland produced a saliva with a kallikrein concentration in the very low range of that regularly seen on sympathetic stimulation.

Urinary kallikrein secretion. The kallikrein concentration in urine was 0.447 ± 0.048 EU/ml and the kallikrein secretory rate was 6.91 ± 2.87 EU/24 h (Table II). The day to day

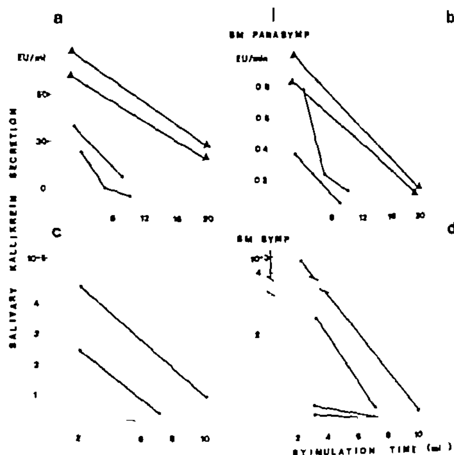


Fig. 2. Salivary kallikrein concentration (a and b) and secretory rate (b and d) during the period of parasympathetic (ductal nerve ● and pilocarpine ▲) and sympathetic (cervical nerve) stimulation of the submandibular gland.

variation for one rat was 14 ±4% for the urinary kallikrein concentration and 32 ±6% for the kallikrein secretory rate.

Kallikrein secretion and its relationship to salivary and urinary volume Kallikrein secretion rate was poorly correlated with salivary flow rate. Best correlation was seen after pilocarpine stimulation of the parotid gland (r = +0 70 P 0 05). The relationship between kallikrein and salivary secretion from one gland could not be estimated, since not enough salivary samples were obtained during the stimulation period

The day to day variations in urinary kallikrein secretory rate and concentration were compared with alterations in urine volume. It was found that individual kallikrein secretory rates were correlated with the urinary fluid volume (r = +0 91 ±0 06, P <0.05). These data were based on 14 rats each providing about 15 measurements. When the mean kallikrein secretory rate for each rat was compared with the mean urine volume, the correlation was r = +0.88 (P <0 05) No relationship was seen between urinary volume and urinary kallikrein concentration

Kallikrein activity in tears Bz Arg-OEt-esterase activity was not detected in tears.

Discussion

In the present study quantification of glandular kallikrein was based on its esterase activity and on its antigenicity. For the esterase measurements N-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) was used as substrate, a method which has proved to give a good estimate of kallikrein activity in rat kidney extracts and urine (Nustad 1970) as well as in rat submandibular gland extracts and saliva (Ørstavik and Gautvik 1977). Moreover quantification of kallikrein by its antigenicity has been shown to be a specific method for kallikrein measurements in rat submandibular gland extracts and saliva (Ørstavik *et al.* 1977. Ørstavik and Gautvik 1977). The enzyme test is more sensitive than the immunological method and was therefore useful for organs with low kallikrein concentration per g wet weight such as the parotid and the exorbital lacrimal glands. Since kallikrein-specific fluorescence has been detected immunohistochemically in the parotid gland (Ørstavik 1978), it is concluded that the esterase activity observed at least partially was due to kallikrein. However in the lacrimal gland neither kallikrein-specific fluorescence (Ørstavik 1978) nor esterase activity was observed. Furthermore, esterase activity could not be detected in tears obtained by stimulation of the exorbital lacrimal gland. It is therefore concluded that kallikrein is lacking or present in extremely low concentrations in this gland. In such organs as the pancreas, where kallikrein is found as a pro-enzyme, immunological kallikrein quantitations could be made (Ørstavik and Glenner 1978).

The innervation of kallikrein-containing cells can be elucidated by studying the secretory pattern of kallikrein. The large amounts of kallikrein found in the rat submandibular gland is almost entirely related to the granular tubular cells (Ørstavik *et al.* 1977) and is most probably located in the secretory granules of these cells (Ørstavik *et al.* 1975). It can thus be deduced that the granular tubules are specifically activated through α-adrenergic sympathetic receptors. The comparably very low secretory rate of kallikrein following β-adrenergic and parasympathetic stimulation indicates that the granular tubular cells are only slightly activated by such fibers. The granular tubular cells have been shown to undergo degranulation when the gland is subjected to -adrenergic sympathetic stimulation but not following β-adrenergic or parasympathetic stimulation (Matthews 1974). These data are in agreement with the above suggested innervation pattern for the granular tubular cells.

It has been shown that pilocarpine in high doses also stimulates the superior cervical ganglion (Schneyer and Hall 1966). However since large amounts of kallikrein are secreted during cervical nerve stimulation, but not during pilocarpine stimulation, it is concluded that the doses of pilocarpine used in the present study did not activate the superior cervical ganglion.

Also in the parotid and sublingual glands the striated ducts seem to be slightly more affected by sympathetic, particularly α-adrenergic, than parasympathetic stimulation. The differences observed in kallikrein secretory rate were small and mostly not significant. However when cervical nerve stimulation was superimposed on pilocarpine stimulation, the kallikrein secretory rate from the parotid gland was shown to increase, confirming that kallikrein secretion is somewhat higher after sympathetic than after parasympathetic stimulation.

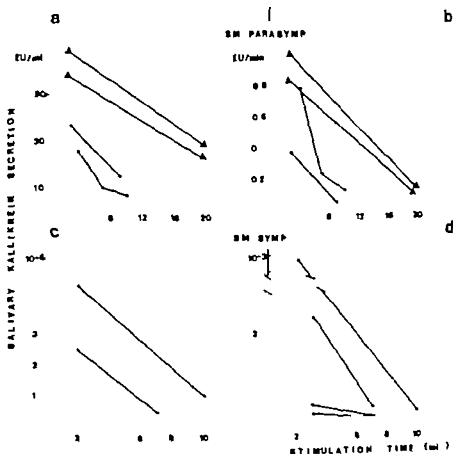


Fig. 4. Salivary kallikrein concentration (a and b) and secretory rate (b and d) during the period of parasympathetic (ductal nerve ● and pilocarpine ▲) and sympathetic (cervical nerve) stimulation of the submandibular gland.

variation for one rat was 14 ± 4 % for the urinary kallikrein concentration and 32 ± 6 % for the kallikrein secretory rate.

Kallikrein secretion and its relationship to salivary and urinary volume. Kallikrein secretion rate was poorly correlated with salivary flow rate. Best correlation was seen after pilocarpine stimulation of the parotid gland ($r = +0.70$, $P < 0.05$). The relationship between kallikrein and salivary secretion from one gland could not be estimated since not enough salivary samples were obtained during the stimulation period.

The day to day variations in urinary kallikrein secretory rate and concentration were compared with alterations in urine volume. It was found that individual kallikrein secretory rates were correlated with the urinary fluid volume ($r = +0.91 \pm 0.06$, $P < 0.05$). These data were based on 14 rats each providing about 15 measurements. When the mean kallikrein secretory rate for each rat was compared with the mean urine volume, the correlation was $r = +0.88$ ($P < 0.05$). No relationship was seen between urinary volume and urinary kallikrein concentration.

Kallikrein activity in tears. Bz-Arg-OEt-esterase activity was not detected in tears.

The kallikrein secretory pattern found in the rat salivary glands resembles that seen in major salivary glands of other species. In the cat submandibular gland sympathetic nerve stimulation resulted in a 90–95% decrease in the total gland kallikrein content (Barton *et al.* 1975). The nerve induced kallikrein release was found to be mediated through α-adrenergic receptors (Gautvik *et al.* 1974). *In vitro* studies on the submandibular gland of the guinea pig showed that kallikrein was released on and β-adrenergic stimulation and also, although less, on parasympathomimetic stimulation (Albano *et al.* 1976). A decrease in salivary kallikrein concentration during the stimulation period was also seen in the cat submandibular gland (Gautvik *et al.* 1974).

The large individual variation in kallikrein content in both glands and saliva and the rather small nerve induced differences observed in the parotid and sublingual salivary kallikrein, indicate that the glandular kallikrein level may be influenced by factors not controlled in the present study. Recent investigations strongly point to a role of mineralocorticoids in the secretion of urinary kallikrein (Geller *et al.* 1972), and such hormones may also influence the secretion of salivary and other glandular kallikreins. In accordance with this concept, glandular kallikreins have been demonstrated in the kidney (Ørstavik *et al.* 1976), in the salivary glands (Ørstavik *et al.* 1975, Brandtzaeg *et al.* 1976, Ørstavik 1978), in the toad bladder (Chao and Margolius 1978), in sweat (Fräki *et al.* 1970), and in feces possibly in part originating from pancreatic pro-kallikrein (Ørstavik and Glenner 1978), whereas in the lacrimal gland kallikrein could not be detected. In these kallikrein-containing organs, electrolyte transport will be influenced by aldosterone (Blair West *al.* 1967 Giebisch 1971). Moreover in the salivary glands and in the kidney the organs in which the cellular localization of kallikrein has been determined (Fig. 3), the kallikrein-containing cells are also known to be the major target cells for mineralocorticoids in these organs (Young and van Lennep 1978, Giebisch 1971).

In the present study   correlation between urinary kallikrein secretion and urine volume, but not between salivary kallikrein secretion and salivary volume, was observed. A relationship between urine water and kallikrein has also been shown in previous studies (Mills and Ward 1975) but has been disputed by others (Margolius *et al.* 1974). The relationship may be due to other physiological processes taking place concomitantly with kallikrein secretion.

In conclusion, salivary kallikrein concentration is highest after sympathetic, particularly α-adrenergic, stimulation in all three major salivary glands of the rat. The kallikrein-rich granular tubular cells of the submandibular gland are specifically activated through α-adrenergic receptors. The distribution of kallikrein in exocrine organs may be related to cells important for the regulation of the sodium/potassium balance.

I want to thank Dr Kjell Nustad for the supply of methkallikrein antiserum and purified kallikreins and for the many helpful discussions.

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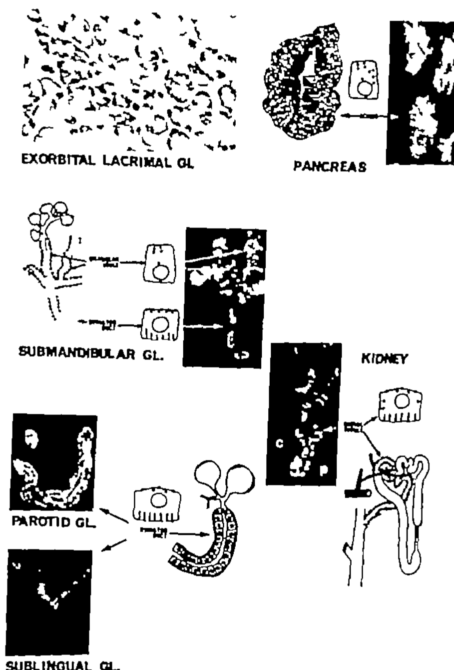


Fig. 3 Immunohistochemical demonstration of the cellular localization of kallikrein in the rat pancreas, kidney and the submandibular, sublingual, and parotid glands. Kallikrein is in all organs, except in the pancreas, found in cells showing infoldings of the basal cell membrane associated with numerous mitochondria, illustrated by the basal striation in the line-drawings. In the pancreas, kallikrein is found as a pro-enzyme probably activated in the duodenum, suggesting that pancreatic kallikrein may have an extra-glandular function. The pancreatic acinar cells resemble the granular tubular cells of the submandibular gland in morphology and by their content of trypsin like enzymes. The exorbital lacrimal gland does not have any cells resembling the salivary striated duct cells, and kallikrein was not found in this gland.

The cellular localization of glandular kallikreins has been published previously: the pancreas in Ørstavik and Glenner (1978), the submandibular and sublingual glands in Ørstavik *et al.* (1975) and Brandtzaeg *et al.* (1976), the parotid gland in Ørstavik (1978), and the kidney in Ørstavik *et al.* (1976). Part of this figure has also been published in Nustad *et al.* (1978).

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The effects of ureteral occlusion and renal venous constriction on kidney kallikrein–kinin and prostaglandin systems in dogs

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Abstract

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The effects of ureteral occlusion and renal venous constriction on kidney kallikrein–kinin and prostaglandin systems in dogs

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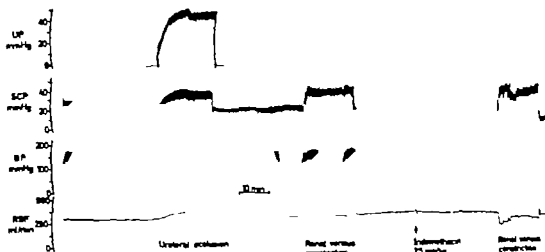


Fig. 1 Continuously registrated parameters in a representative experiment. UP = ureteral pressure, SCP subcapsular pressure BP = arterial blood pressure and RBF = renal blood flow

by extreme reductions of urinary kallikrein excretion (Bevan *et al* 1974 Keiser *et al.* 1976) Loop diuretics and renal artery constriction produce marked, yet opposite effects upon the intrarenal pressure Consequently this has led to the hypothesis that urinary kallikrein excretion might be influenced by the intrarenal pressure (Mills *et al* 1976, Olsen and Anfelt Rønne 1976) One of the objects of the present study was to investigate this hypothesis in anesthetized dogs, in which the intrarenal pressure was comparably raised by ureteral occlusion or by renal venous constriction The later procedure has previously been reported to enhance the appearance of kinins in urine (Alzamora and Capelo 1973) Therefore urine kinin excretion was also measured in the present study in order to investigate if a relationship could be demonstrated between acute changes of urine kallikrein and kinin excretions. Finally indomethacin was used as a tool to investigate the possible relationship between renal prostaglandins and the kallikrein–kinin system. The last issue was prompted by a previous report which showed that indomethacin pretreatment in dogs impaired the enhancements of urinary kallikrein excretions provoked by the administration of the loop diuretic bumetanide (Olsen and Ahnfelt Rønne 1976).

Methods

6 female mongrel dogs (16–28 kg) which had been fasted vernight were used for the experiments. The dogs were kept on a standard diet (Doggy®) with free access t w ter

The dogs were anesthetized with pentobarbital sodium (30 mg/kg i.) and intubated for spontaneous respiration. A Statham (P 23 Db) transducer was connected to carotid artery for blood pressure measurements, and a catheter was placed in the jugular ein fo subsequent infusion. The abdomen as opened by midline incision. The ureters were cannulated for urine collections, and alve placed at the *tip* of the cannula in the left ureter The valve was constructed so that the ureteral pressure could be raised to the desired pressure level of 40–50 mmHg (registered by a Statham pressure *transducer*) before an urine overflow was established. A flow probe was placed around the left *renal artery* for the measurement of renal blood flow (electromagnetic flow meter Nycot on®). A thread sling for manual constriction was placed around the left renal vein and the subcapsular (intrarenal) pressure as measured by a P 19 miniature transducer (Konigsberg *Instr*). Blood pressure renal blood flow and subcapsular

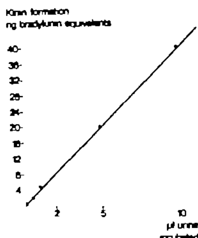


Fig. 2. Relationship between amount of urine incubated and kinin formation. Normal urine was pooled from 4 dogs. The symbols ○ and ● represent two separate series of incubations in which kinins have been determined on different isolated rat uteri.

pressure was continuously recorded on a polygraph. At the end of the experiment the dog was killed by lethal dose of pentobarbital. The subcapsular pressure recorded at this stage was used as zero value.

Following completion of surgery the dogs were loaded with 100 ml 10 % mannitol in saline (10 ml/min) and 2 ml/min was infused throughout the experiment. Urine was collected on ice for 15 min periods. Fig. 1 shows the recordings from an experiment. When urine flow had stabilized 30–45 min after surgery control urine was collected. The urine flow from the left kidney was then stopped until the ureteral pressure rose to 40–50 mmHg. The initial 1–2 ml urine was discarded and subsequently 15 min urine overflow was collected at this pressure. Following 15 min of recovery control urine was again collected. The left renal vein was then constricted to raise the subcapsular pressure to the same level as that obtained during ureteral occlusion, and urine was collected for 15 min. After recovery indomethacin (2 mg/kg) was administered i.v. Control urine was collected 15 min later and the procedure with constriction of the renal vein was repeated as before the administration of indomethacin. Urine collected from the right and untouched kidney served as an additional control to establish the effects of time and indomethacin on kidney function.

The urine samples were immediately diluted with ice cold distilled water ad 30.0 ml, in order to provide an uniform and suitable volume for subsequent extractions of prostaglandins and kinins.

During the elevations of the intrarenal pressure to 40–50 mmHg by renal vein constriction proteinuria (uristix) was observed in 4 of the experiments, but all samples were free of blood. Hematuria was, however, occasionally observed in preliminary experiments when the vein was constricted to raise the intrarenal pressure to more than 50–60 mmHg.

Analyses

Urine kallikrein excretion. Kininogenase activity in urine was determined by the micromethod of Marin-Grez and Carretero (1972), which was slightly modified. In addition to kallikrein urine contains other kininogenases, yet less active (*e.g.* plasmin, uropepsin). In order to enhance the specificity of the kallikrein measurement, the heated (60°C for 3 hr) dog plasma containing kininogen was obtained from 9 ml dog venous blood collected on 1 ml aprotinin (10 000 KIE/ml Trasylol® Bayer), a protease inhibitor that does not inhibit dog urine kallikrein (Morwalu *et al.* 1976). The kinin forming incubations were then performed as follows: 200 µl buffer (0.1 M phosphate, pH 7.4) diluted urine (corresponding the urine formed in 1/100 min) plus 100 µl Tyrode' solution were incubated with 50 µl heated dog plasma in plast tubes for 2 min at room temperature (22°C). The kinin forming reaction was stopped by placing the tubes in boiling water for 5 min, and the kinins released were subsequently assayed on the isolated rat uterus (Olsen and Ahnfelt-Rønne 1976). Fig. 2 shows that the kinin formation was directly proportional to the amount of urine incubated.

The incubated samples from each experiment were assayed on the same uterus with reference to synthetic bradykinin (Sandoz). Differences in kallikrein activity among samples exceeding 20 % were safely detected. One unit of kallikrein is the amount of enzyme which forms 1 µg of bradykinin equivalent by the incubation procedure.

Urine prostaglandin excretion The ice cold urine samples (30 ml) were acidified (pH 3) with 5 formic acid and lipids were extracted with an equal volume of chloroform The water phase was frozen and stored for subsequent extraction of kinins. Polar lipids (*i.e.* prostaglandins) were purified by silicic acid chromatography (Olsen *et al* 1976) and the biological activity was assayed on the isolated rat fundus strip in terms of PGE_2-like activity (Olsen *et al.* 1976). Recovery of H PGE_2 added to the urine was more than 80 . The identity of the PGE-like material was established in three of the experiments in which the samples were pooled after assay 10 000 cpm H PGE_2 was added and lipids were reextracted and applied on silica coated plates. The plates were developed in the solvent system chloroform methanol acetic acid water (90 9 1 0 65) and the H PGE_2 spot was precisely located in a Spark chamber After zonal division the plates were scraped off and it was confirmed that the biologically active material (rat fundus strip) cochromatographed with the H PGE_2 standard.

Urine kinin excretion A possible enzymatic formation of methionyl-lysyl-bradykinin during the acidic extraction procedures (Hial *et al* 1976) was avoided by the following steps. 1) Urine was maintained cold during acidification and lipid extraction and urine (water phase) was subsequently transferred to 100 ml plast flasks and stored frozen (Miwa *et al.* 1969). 2) In 3 of the experiments the acidic protease inhibitor pepstatin (Beckman) was added (50 μg/sample) (Hial et al. 1976). The result of these experiments were essentially similar to those in which pepstatin was not used. Furthermore it should be noticed that the rat uterus, which was used for the assay procedure (see below), is less sensitive to methionyl-lysyl-bradykinin than to bradykinin or kallidin.

Urine kinins were extracted by batch absorption to Amberlite CG-50(H) (100–200 mesh) (Miwa *et al.* 1969 Hial *et al* 1976). After thawing the pH of the water phase of urine was adjusted to 4 0 and was shaken with 100 mg resin in plast flasks for 2 h. The resin was settled by standing and was transferred to plast tubes. After repeated washing with distilled water the kinins were eluted with 2 ml 9M acetic acid by shaking the resin for 1 hour The resin was sedimented by centrifugation and the acetic acid was transferred to 5 ml tubes and was evaporated at 60°C in vacuo. Kinins were redissolved in 0.5 ml 0.9 NaCl plus 50 μl 2M Tris. Recovery of ^{3}H-bradykinin added as internal tracer in eighteen samples from different experiments was 59 ± 3 Approximately 10 loss of the kinins occurred during the initial extraction of prostaglandins. Kinins were assayed on the isolated rat uterus (Olsen and Ahnfelt Rønne 1976). The test samples behaved like bradykinin on the tissue and were free from interfering substances. In addition the samples produced vasodilation in the dog hind limb blood flow preparation (Nasjletti *et al* 1975) and the biological activity was destroyed by carboxypeptidase B and by chymotrypsin, but not by trypsin, which in contrast enhanced the biological response of the rat uterus. These biological criteria are characteristic of kinins (Shaw and Ramwell 1968 Nasjletti *et al* 1975) The identity was finally established utilizing ^{3}H bradykinin as a tracer The samples were pooled after assay and kinins were reextracted. The acetic acid eluate was concentrated and was applied on aluminiumoxid precoated plates (DC Alufolien, Aluminiumoxid F254 Neutral, Typ T Merck). The plates were developed in the solvent system butanol: water acetic acid (25 12 2, the organic layer). H bradykinin was precisely located in the Spark chamber and appeared in one distinct spot with $R_f = 0.14$–0.19 Kallidin and methionyl-lysyl-bradykinin were run separately R_f of kallidin was 0.06–0.09 and methionyl-lysyl bradykinin mobilized in front of kallidin. After zonal division the plates were scraped off into plast tubes and kinins were eluted with Tyrodes solution. The biologically active materials (rat uterus) cochromatographed with bradykinin and kallidin zones. Other activity was absent or negligable

Urine sodium and creatinine excretions. Sodium was determined by flame photometry and creatinine by Jaffe's reaction.

Statistics. The results are presented as mean ± S E. The statistical significance of the results was assessed by Student's t test after a logarithmic transformation of paired (ratio) data.

Results

Fig 1 shows the continuously registrated parameters in a representative experiment The elevations of the ureteral pressure to a steady level of 46 ± 1 mmHg were parallelled by similar and not significantly different elevations of the subcapsular pressure to 45 ± 3 mmHg. Table I summarizes the results of the measurements on the experimental left kidney and Table II shows the results of the control kidney

Table I. The effect of ureteral occlusion and renal venous constriction on kidney parameters. Results are mean ± S.E. (N = 9). Significance was assessed by paired comparison. = $p < 0.05$, = p 0.01 $p < 0.001$, n.s. = non-significance. ★ = significance at $(1/2)^8$ 1.5% level by non-parametric method. § below the sensitivity of the assay procedure

[illegible]	Control I	Ureteral occl. II	II/I	Control (Recovery) III	III/I	Venous const. IV	IV/III	IV/II	Control after indometh. V	V/III	Venous const. VI	VI/V	VI/IV
Femoral pressure, mmHg	145±6	145±6		143±6	n.s.	146±6	n.s.	n.s.	144±7	n.s.	146_6	n.s.	n.s.
Renal blood flow, ml/min	153_39	187±50		172±43	n.s.	170±44	s.	n.s.	168±47	s.	114±38		
Subcapsular pressure, mmHg	28 3	45_3		26±4	n.s.	47±3		s.	17±2		48±4		n.s.
Urine flow, ml/min	1.3±0.2	0.8±0.1		1.5±0.1	n.s.	1.1±0.1		n.s.	1.5±0.2	n.s.	0.7±0.1		
Sodium excretion, μeq/min	89±23	26±8		98±27	s.	64±24	n.s.		89±39	n.s.	38±13	s.	
Creatinine excretion, μmol/min	2.8_0.2	2.2±0.2		2.7±0.2	s.	2.4±0.2		n.s.	2.3±0.3		1.3±0.3		
Kallikrein excretion, U/min	0.7_0.2	0.7_0.2		0.7±0.2	n.s.	0.4±0.1	n.s.	n.s.	0.7±0.2	n.s.	0.2±0.1		
Kinin excretion, ng/min	0.4±0.1	0.4_0.1		0.8±0.3		5.3±2.3	★	★	1.2±0.4	n.s.	4.1±1.6	★	n.s.
PG(E) excretion, ng/min	0.4 0.1	0.8±0.2		0.4±0.1	n.s.	1.1±0.3		s.					

Before indomethacin treatment

Table I and Fig. 1 show that comparable enhancements of the intrarenal pressure (subcapsular pressure) by ureteral occlusion or by renal vein constriction exhibit different hemodynamic and excretory effects. The enhanced renal blood flow and the reduced creatinine excretion (glomerular filtration rate) induced by ureteral occlusion indicate a decrease of the filtration fraction by approximately 35%. During renal venous constriction renal blood flow did not change significantly and the filtration fraction only decreased by approximately 9%.

When the intrarenal pressure was enhanced by ureteral occlusion or by renal venous constriction urine volume and sodium excretion were reduced. The percent decrease of sodium excretion generally exceeded that of creatinine excretion, and consequently fractional sodium excretions fell (Table I). This antinatriuresis was significantly more pronounced during ureteral occlusion than during renal venous constriction in which condition the changes of sodium excretion were very variable (range: 148% increase – 81% decrease).

In control urine collections (Table I and II) urine kallikrein excretion appeared to be very constant with changes less than 0.1 U/min among successive periods. Ureteral

TABLE II The effect of indomethacin and time on control kidney parameters. Results are mean ± S.E. (N = 6). Significance was assessed by paired comparison. a = $p < 0.05$, n.s. = non-significance. Indomethacin (2 mg/kg) was administered i.v. at time o.

Min	−100–85	−50–35	−15–0	0–15	15–30
Urine flow ml/min	1.2 ± 0.1	$1.4 \pm 0.2^{n.s.}$	$1.5 \pm 0.2^{n.s.}$	1.4 ± 0.2^{a} n.	1.2 ± 0.1
Sodium excretion, µeq/min	75 ± 22	95 ± 38 n.	110 ± 43 n.	100 ± 39^{a}	67 ± 29^{a}
Creatinine excretion, µmol/min	2.9 ± 0.3	$2.8 \pm 0.2^{n.s.}$	2.6 ± 0.3	2.5 ± 0.3 n.	2.3 ± 0.3
Kallikrein excretion, U/min	0.6 ± 0.1	$0.6 \pm 0.1^{n.s.}$	0.6 ± 0.1 n.	0.6 ± 0.1 n.	0.6 ± 0.1
Kinin excretion, ng/min	2.2 ± 1.4	$2.1 \pm 1.4^{n.s.}$	2.2 ± 1.4 n.	1.3 ± 0.3	1.0 ± 0.3^{a}

occlusion did not significantly affect the kallikrein excretion, which, however was reduced in 4 of 6 expts. during renal vein constriction. The latter procedure was associated with marked yet variable enhancements of urine kinin excretion (range 0.5–13.3 ng/min) which remained unchanged during ureteral occlusion.

Renal venous constriction and ureteral occlusion similarly enhanced urinary excretion of E type prostaglandins.

In control periods it was not possible to assess any significant correlation between urine kallikrein and kinin excretions or between these parameters and other hemodynamic or excretory functions. A marked correlation (r = 0.92, N = 6) was assessed between sodium excretion and kinin excretion in the collections during renal venous constriction (Fig. 3).

After indomethacin treatment

The administration of indomethacin decreased the urinary excretion of prostaglandins (Table I). In addition a significant reduction of the subcapsular pressure was observed although renal blood flow and urine flow were unchanged. In one right control kidney with extremely high yet constant kinin excretion of 8.9 ng/min before the administration of indomethacin this compound produced a prompt reduction of the kinin excretion to 2.3 ng/min. Otherwise, the effects of indomethacin on the parameters were small, and it cannot

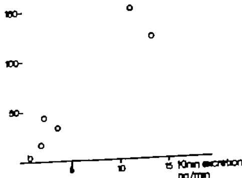


Fig. 3 Relationship between kinin excretion (abscissa) and sodium excretion (ordinate) during ureteral occlusion (●) and renal venous constriction (O).

be ruled out that they were related to time rather than to inhibition of prostaglandin synthesis.

The kinin excretion increased when the renal vein was constricted after the administration of indomethacin as also observed before the administration of indomethacin, and the urinary kallikrein excretion decreased to approximately 30% of control.

Constriction of the renal vein after inhibition of prostaglandin synthesis resulted in a marked reduction in renal blood flow (Fig. 1) and creatinine excretion (glomerular filtration rate), and a decreased urine flow and sodium excretion (Table I). Nevertheless, in 5 of 6 expts in this condition the sodium excretion was still higher than the sodium excretion observed during ureteral occlusion before the administration of indomethacin.

Discussion

The results show that intrarenal pressure elevation by renal venous constriction increased urine kinin excretion in association with unchanged or even markedly reduced excretion of urinary kallikrein. A comparable enhancement of the intrarenal pressure by ureteral occlusion did not affect urinary kallikrein or kinin excretions. The present results therefore failed to support the hypothesis that urinary kallikrein excretion is directly related to the intrarenal pressure (see introduction).

The enhanced urinary kinin excretion during renal venous constriction observed in the present experiments agree with results previously reported by Alzamora and Capelo (1973), and probably reflects an increased intrarenal kinin formation. This can hardly be due to increased kallikrein release, since urinary kallikrein excretion was unchanged or actually decreased. More likely the change in kinin excretion could be a consequence of the enhanced hydrostatic capillary pressure. This might increase the microvascular surface area and produce an edema like condition with increased outward movement of fluid in association with increased permeability to proteins i.e. kininogens. At modest elevations of the intrarenal pressure by ureteral occlusion or by renal venous constriction lymph and lymph protein flow increase almost proportionally in rats (Källskog and Wolgast 1975) and dogs (Haddy *et al.* 1959, Le Brie and Mayerson 1960, Mayerson 1963). When the venous pressure level in dogs exceeds 40 mmHg a disproportional increase of lymph protein flow has been reported, and protein concentration in lymph markedly increased (Haddy *et al.* 1959, Le Brie and Mayerson 1960). This might suggest that the availability of kininogen rather than kallikrein might be the factor which limits the rate of intrarenal kinin formation. Consequently it appears that urinary kallikrein excretion is not a reliable indicator of the intrarenal kinin activity under conditions of renal venous constriction. Neither did we observe any correlation between kallikrein and kinin excretions in the present control periods.

Indomethacin treatment did not significantly affect the control urine kallikrein excretion under the present experimental conditions. A reduction in urinary kallikrein excretion observed during renal venous constriction was, however, potentiated by the administration of indomethacin. Inhibition of prostaglandin synthesis by indomethacin in dogs has previously been reported to impair the enhancement of urinary excretions of kallikrein

which follows the cessation of renal intraarterial infusion of noradrenaline (Mills and Obika 1977) or the administration of bumetanide (Olsen and Ahnfelt Rønne 1976). This opens up the possibility that regulation of renal kallikrein release may be influenced by local prostaglandin synthesis. The present data might suggest that kinins or the adjacent peptide fragments (Oh-ishi *et al* 1977) exhibit a negative feed-back regulation on kallikrein release, perhaps similar to that which exists between angiotensin II and renin and it is possible that prostaglandins suppress this feed-back inhibition. Alternatively the reductions of urinary kallikrein excretion during renal venous constriction after indomethacin treatment might be secondary to the marked reductions of renal blood flow and/or urine flow (Bevan *et al* 1974 Keiser *et al.* 1976, Mills *et al* 1976)

Intrarenally generated kinins have been suggested to be natriuretic hormones, and rats treated with antibradykinin serum showed a reduced ability to excrete a sodium load (Marin-Grez 1974) With obvious reservations due to the low number of data the present results might support the concept that kinins exert a natriuretic function since a significant correlation between sodium and kinin excretions was observed during enhancement of the intrarenal pressure by renal venous constriction.

According to Poiseuille's law the increased outflow resistance induced by renal venous constriction should be accompanied by proportional decrements of renal blood flow all other factors being equal. The unchanged renal blood flow might therefore result from a compensatory vasodilation (Kishimoto *et al* 1972) Renal intraarterial infusion of kinins in dogs produces vasodilation and increases the appearance of prostaglandin E in blood and urine (McGiff *et al* 1972, Olsen 1978 a) In the present experiments renal venous constriction and ureteral occlusion increased urinary prostaglandin excretion to a similar degree and produced renal vasodilation. An increased kinin excretion was only induced by renal venous constriction Consequently intrarenally generated kinins cannot be the only factor responsible for the vasodilation and the prostaglandin synthesis under these conditions, if involved at all.

The increased urinary excretion of prostaglandins during ureteral occlusion or renal venous constriction is presumably mainly a result of the elevation of the intrarenal pressure. We have previously reported comparable enhancements of urinary prostaglandin excretions during similar elevations of the intrarenal pressure by ureteral occlusion and by kidney compression in anaesthetized dogs (Olsen *et al* 1976) Furthermore an intrarenal pressure dependent mechanism of prostaglandin release has been demonstrated in the isolated Krebs-Henseleit perfused rabbit kidney (Olsen 1978 b) The prostaglandins released by such a pressure mechanism have been implicated in the accompanying vasodilation, since indomethacin treatment inhibited the vasodilation during ureteral occlusion or during kidney compression and caused the glomerular filtration rate to decrease severely (Olsen *et al* 1976). This was also observed in the present experiments in which indomethacin treatment impaired the vasodilation observed during renal venous constriction, and this was accompanied by decreased glomerular filtration rate (creatinine excretion) Presumably the vasodilations observed during elevation of the ureteral pressure or during renal venous constriction are mediated by prostaglandins, and both procedures have been reported similarly to redistribute the cortical blood flow toward the inner cortex (Abe *et al* 1973).

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Adenosine and cyclic AMP in cerebral cortex of rats in hypoxia, status epilepticus and hypercapnia

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Abstract

REHNCRONA, S., B. K. SIESJÖ and E. WESTERBERG. *Adenosine and cyclic AMP in cerebral cortex of rats in hypoxia, status epilepticus and hypercapnia.* Acta physiol. scand. 1978. 104. 453–463.

The influence of hypercapnia, hypoxia and status epilepticus on cerebral cortex concentrations of adenosine, adenine nucleotides and cyclic AMP was studied in lightly anaesthetized (70% N_2O) and artificially ventilated rats. Neither hypercapnia (arterial P_{CO_2} about 80 and about 300 mmHg) nor hypoxia (nominal values of 19 mmHg) altered tissue concentrations of AMP, cyclic AMP or adenosine. Bicuculline-induced status epilepticus was accompanied by increased concentrations of cyclic AMP but adenosine concentration did not change. Experiments with ischemia, and those in which tissue hypoxia was exaggerated by unilateral carotid artery ligation, showed that tissue adenosine concentrations were elevated only when AMP concentrations rose. It is concluded that the marked increase in cerebral blood flow which occurs in hypoxia and status epilepticus is unrelated to changes in tissue adenosine concentration and that the increase in cyclic AMP during neuronal hyperactivity is triggered by other mechanisms than adenosine accumulation.

Recent results suggest that adenosine may be one important factor mediating vasodilatation in the brain. The evidence is based on two main lines of results. First, at least when sufficiently pronounced, conditions associated with vasodilatation in the brain lead to dephosphorylation of AMP with accumulation of adenosine and translocation of adenosine to cerebral extracellular fluids. These conditions include electrical stimulation (Pull and McIlwain 1972, 1973, Rubio *et al.* 1975) and reduction of oxygen tension in brain secondary to hypoxia or ischemia (Pull and McIlwain 1972, Rubio *et al.* 1975). Second, when adenosine is applied to pial vessels, dilatation occurs (Berne *et al.* 1974, Wahl and Kuschinsky 1976). In view of such results it has been suggested that release of adenosine occurs whenever there is an increase in functional activity or a restriction of oxygen supply, and that adenosine mediates vasodilatation by its extracellular intracerebral effect on resistance vessels (Rubio *et al.* 1975).

Our own previous results in hypoxia and in epileptic seizures appeared inconsistent with the above hypothesis. Thus, hypoxia leads to pronounced vasodilatation without measurably affecting the tissue concentration of AMP (Bachelard *et al.* 1974, Norberg and Siesjö 1975, Johansson and Siesjö 1975), and, in bicuculline-induced status epilepticus, there is little or

no increase in AMP concentration in spite of marked increase in blood flow (Meldrum an Nilsson 1976, Chapman *et al* 1977). Since the 5 nucleotidase reaction, which forms adeno sine from AMP seems to be mainly determined by substrate availability (Kluge *et al* 1972; increase in tissue concentration of adenosine seemed less likely

We recently developed a sensitive chromatographic technique for measuring adenosin concentration in tissue (Nordström *et al* 1977). Results obtained showed that, in ischemia there was a strict correlation between increase in AMP and adenosine In the present study we have examined tissue concentration of adenosine in situations accompanied by increase cerebral blood flow Changes in adenosine concentrations were correlated to tissue concen trations of lactate and pyruvate as well as of labile phosphates, including cyclic AMP Preliminary accounts of some of the findings have been published (Rehncrona *et al* 1977 Nilsson *et al* 1977)

Methods

Operative and sampling technique

Male Wista rats of a S.P F stra n were anaesthetized with 2 3% halothane, tracheotomized, immobilized with tubocurarine chloride (0.5 mg kg^{-1}), and ventilated with 70% N_2O and 30% O_2 to yield an arterial P_{CO_2} of 35–40 mmHg. After tracheotomy halothane supply was discontinued. Catheters were placed in a femoral artery for lectromanometric blood pressure recording, and for anaerobic sampling of blood, and in a femoral vein for i jections. Body temperature was maintained close t 37°C. In all animals, the skin over the kull bone was incised to accomodate plastic funnel for later freezing of the brain tissue *in situ* (Pontén *et al* 1973). This tech ique gives relatively slow freezing of the tissue but, since the circulation is not interrupted prior to freezing, autolytic artefacts seem to be avoided (see also N lsson *et al.* 1975).

Experimental conditions

Arterial *hypoxia* was induced by reducing the O concentration of the insufflated gas mi ture the N_2O concentration being kept const nt by admixture of N_2. In some experiments, the right common carotid artery was occluded with a rubber clamp after that the cervical sympathetic trunc and the vagus nerv had been dissected free of the vessel. *Status epilepticus* was nduced by i.v. injection of bicuculline in a dose of 1.2 mg kg^{-1} (see Chapman *et al* 1977) In these animals, the EEG was continuously recorded. *Hypercapnia* was induced by adding either 10 or 40 CO to the insufflated gas mixtures, with corresponding reductions in N_2O concentration.

Analytical techniques

Arterial P_{O_2}, P_{CO_2} and pH were measured t 37 C with microelectrodes (Eschweiler and Co, Kiel, and Radiometer Copenhagen), w th due corrections for deviations of body temperature from 37°C.

Followi g freezing *in sit* the bra was chiselled ut at liquid trogen temperature and stored t 80 C until analysis. Cortical tissue from front parietal region w dissected, weighed and extracted ith HCl-Methanol at −22°C (see Folbergrová *et al* 1972) F rther extraction (with perchloric acid) was carried out at 0°C and following centrifugation, the neutral zed e tracts were used for metabolite analyses. Concentrations of phosphocreatine (PC), ATP ADP AMP lactate nd pyruv te were measured w th the enzymatic fluorometric techniques of Lowry and Passonneau (1972) as described in a previous communication (Folbergrová *et al.* 1972). Cyclic AMP was measured with a protei binding technique based on the methods described by Gilman (1970) nd Brown *et al* (1971), using commercial kit (The Radiochemical Centre, Amersham, England). Interference with the measurements were excluded by using e traction blanks and internal standards, run together with the tissue extracts (Folbergrová 1975). Adenosine was measured w th high perf rmance liquid chromatography (Waters, model U6K Universal Injector 6000 A Solvent Delivery system, 440 UV Absorbance Detector) a described previous article (Nordström *et al.* 1977). In all ex perimental situations, brains were analysed together w th ppropriat control bra ns. Adenosine concentrations were evaluated usi g several standard samples added t e traction blanks.

Calculations

The energy charge of the adenine nucleotide pool was calculated according t Atkinson (1968). Statistical d fferences were evaluated with the Student's *t* test

Results

Control values

In the material published previously (Nordström *et al* 1977) control adenosine concentrations were close to 1 µmol kg⁻¹ In the present study control values obtained ranged from 0.3 to 1 µmol kg⁻¹ The cause of this variability is not apparent but since re-analyses of extracts gave values consistent with previous results the variability seemed to represent true differences in adenosine concentration. Whatever is the explanation the variability does not affect the results of the present study since experimental brains were analysed together with appropriate controls.

Complete ischemia

In order to assess the rate of adenosine formation in cerebral cortex during anoxia, and the maximal values obtained, rats under 70% N_2O were decapitated and tissue was frozen for analysis after 1 5 or 15 min. In the latter two groups, the brains were kept in a water bath at 37°C before freezing Fig. 1 shows that, during the first 5 min, adenosine concentrations rose at a rate of 50–80 µmol kg⁻¹ min⁻¹ After 15 min, tissue concentrations were close to 550 µmol kg⁻¹

Hypercapnia

Results reported by Rubio *et al.* (1975) indicate that there is an inverse relationship between tissue P_{CO_2} and adenosine concentration. In the present experiments, arterial P_{CO_2} was increased to either 85 or to 300 mmHg for 30 min arterial P_{O_2} was kept at 100–140 mmHg. Mean arterial blood pressure ranged from 150–160 mmHg in all groups. As Table I shows, hypercapnia failed to alter tissue concentrations of AMP cyclic AMP or adenosine. There were no signs of energy failure and the decrease in phosphocreatine (PCr) is explained by a pH dependent shift in the creatine phosphokinase equilibrium (see Siesjö *et al* 1972) The decrease in pyruvate and lactate could be explained by decrease in glycolytic flux (Folbergrová *et al.* 1975).

Hypoxia

In the initial series, arterial P_{O_2} was reduced to 23–25 mmHg for 1 and 2 min, respectively The arterial P_{CO_2} was kept t 35–40 mmHg and arterial pH was between 7.35–7 45 There was no significant fall in blood pressure. Table II shows that this degree of hypoxia was associated with expected changes in lactate, pyruvate, PCr and ADP (*cf* Bachelard *et al* 1974 Norberg and Siesjö 1975). There was a slight increase in cyclic AMP after 1 min, but the suggested minor elevation of adenosine after 1 and 2 min of hypoxia was not statistically significant.

In order to further explore changes in adenosine concentration in hypoxia, three additional series of experiments were performed. I the first, arterial P_{O_2} was reduced in steps and the tissue as frozen at the end of a 15 min hypoxic period Fig. 2 show that, at Pa_{O_2} of 35 mmHg, there was a rise in lactate concentration (and lactate/pyruvate ratio not shown) and, at Pa_{O_2} 25 mmHg, there was fall in PCr concentration as well. However ATP ADP

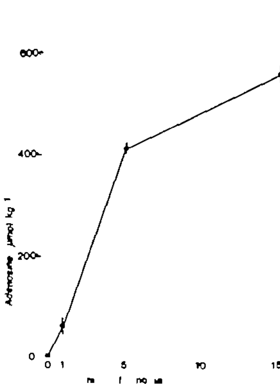


Fig. 1

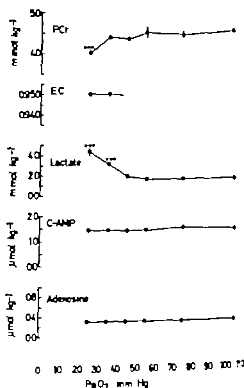


Fig. 2

Fig. 1 Rate of adenosine formation in rat cerebral cortex during anoxia. Each group consists of 3 animals, decapitated after a 30 min steady state period on 70% nitrous oxide anaesthesia with normal arterial blood gases. The brains were kept at 37°C for varying periods before freezing with liquid nitrogen. The brains of control animals (0 min of anoxia, n = 10; adenosine = 0.9 ± 0.1 μmol kg^{-1}) were frozen with liquid nitrogen *in situ*. Values are means ± S.E.

Fig. 2. Concentrations of phosphocreatine (PCr), lactate, cyclic AMP and adenosine in rat cerebral cortex after 15 min at different arterial oxygen tensions (Pa_{O_2} = 107 ± 3, 73.3 ± 0.9, 54.1 ± 0.6, 43.6 ± 0.7, 35.0 ± 0.5 and 26.7 ± 0.5 mmHg, respectively). Adenylate energy charge (E.C.) was calculated as (ATP + 0.5 ADP)/(ATP + ADP + AMP). Each group consists of 4 animals. Values are means ± S.E. *** = $p < 0.001$

and AMP (not shown), as well as the calculated adenylate energy charge remained unchanged and neither adenosine nor cyclic AMP concentrations were altered. In 3 control animals and in 5 animals subjected to a 15 min period of hypoxia (Pa_{O_2} ≈ 25 mmHg), cerebrospinal fluid (CSF) was sampled from cisterna magna and immediately frozen in liquid nitrogen. Mean CSF concentrations of adenosine in the hypoxic and control groups were 0.2 ± 0.1 (mean ± S.E.), and 0.3 ± 0.3 μmol kg^{-1}, respectively. Thus the results failed to show that extracellular adenosine concentrations were elevated in hypoxia.

In the second series, arterial P_{O_2} was reduced to about 20 mmHg and the tissue was frozen after 2 min. As Table III shows, this degree of hypoxia was sufficient to alter tissue concentrations of lactate, pyruvate, PCr and ADP and there was a small reduction in adenylate energy charge (*cf.* Norberg and Siesjö 1975). However, there were no changes in concentrations of AMP, adenosine or cyclic AMP.

In the third series, arterial P_{O_2} was reduced to 20–25 mmHg in animals in which the right common carotid artery had been ligated. Table IV shows that in normoxic controls the

TABLE I Concentrations of phosphocreatine (PCr) adenine nucleotides (ATP ADP AMP) lactate pyruvate (mmol kg^{-1}), as well as of cyclic AMP and adenosine (μmol kg^{-1}) in rat cerebral cortex after 30 min of hypercapnia induced by ventilating the animals with 10 or 40% CO_2.

	Control 4	10 CO_2 −4	40 CO_2 4
P_{CO_2}	41±1	86±5	305±3
pH (blood)	7 40±0 03	7 13±0 02	6 68±0 06
PCr	4 64±0.10	3.94±0.12	2 36±0 07*
ATP	3 00±0 03	3 05±0 02	3 05±0 03
ADP	0 243±0 009	0.248±0 004	0 260±0 007
AMP	0 039 _0 001	0 039±0 001	0 043±0.002
E.C.	0 951±0 001	0 951±0 000	0 949±0 001
Lactate	1.70±0.10	0 90±0 07*	1.03±0.13**
Pyruvate	0.137±0 009	0.054±0 003 **	0 021±0 001
cAMP	1 66±0 03	1 48±0.11	1 62±0 04
Adenosine	0 6±0 0	0.7±0.1	0 6±0.1

The arterial oxygen tension was kept between 100–140 mmHg in all groups. Adenylate energy charge (E.C.) was calculated as ATP 0 5 ADP/(ATP + ADP AMP). Values are means ± S.E. p 0 05, −p 0 01 *** p 0 001

spared side had values for labile metabolites that were close to normal (one animal was excluded since there was reduced ATP and PCr and increased ADP and AMP concentrations. In this animal, adenosine concentration was 2.6 μmol kg). In hypoxic animals, adenosine concentrations rose in 4 out of 6 animals to 17 104 107 and 140 μmol kg^{-1} respectively However this increase was correlated t a derangement of cerebral energy state with markedly increased AMP concentrations. Interestingly enough, cyclic AMP concentrations were increased (above μmol g^{-1}) only in those three animals in which adenosine concentrations rose above 100 μmol kg

TABLE II Concentrations of phosphocreatine (PCr), adenine nucleotides (ATP ADP AMP), lactate, pyruvate (mmol kg^{-1}), the calculated adenylate energy charge (E C.), and lactate/pyruvate (La/Py) ratio, as well as the concentrations of cyclic AMP and adenosine (μmol kg^{-1}) in rat cerebral cortex after short periods (1 and 2 min) of hypoxia

	Control (n 4)	Hypoxia 1 min (n = 4)	Hypoxia 2 min (n 4)
P_{O_2}	134 4 7	22.5±1 6	25.2±1 0
PCr	4 51 _0.10	3 70±0.15	3.87±0.14**
ATP	2 97±0 04	2 93±0 03	2 93±0 02
ADP	0 278±0 002	0 312±0 006**	0 291±0 004*
AMP	0 038 _0 001	0 037±0 001	0 038±0.002
EC	0 946+0 000	0 941±0 001	0 944±0 000*
Lactate	1 58 0 06	3 05±0 19*	3 21±0.20***
Pyruvate	0.116±0 008	0 156±0 005	0 168+0 007*
La/Py	13 7 0 67	19 6 _1 28*	19 1±0 57*
C-AMP	1.34±0 03	1 50 _0 06	1.45+0 06
Adenosine	1 1±0.1	1 3±0 2	1.5±0 1

Values are means ± S E. p 0 05, ** p 0 01 p 0.001

TABLE III Concentrations of phosphocreatine (PCr), adenine nucleotides (ATP ADP AMP), lactat pyruvate (mmol kg⁻) the calculated adenylate energy charge (E.C.) and lactate/pyruvate ratio as well as the concentrations of cyclic AMP and adenosine (μmol kg^{-1}) in rat cerebral corte after min of pronounced arterial hypoxia (Pa_{O_2} < 20 mmHg).

	Control (n = 6)	Pronounced hypoxia 2 min (n = 6)
Pa_{O_2}	116 ± 3.8	19 0 ± 0.3
PCr	4 42 ± 0.06	3.39 ± 0.05*
ATP	3.00 ± 0 02	3 00 ± 0.01
ADP	0.285 ± 0.004	0.305 ± 0 002
AMP	0 049 ± 0.001	0.047 ± 0.001
E.C.	0.943 ± 0.001	0.940 ± 0.000
Lactat	1.60 ± 0 07	5 40 ± 0.20*
Pyruvate	0.123 ± 0 004	0.225 ± 0.007*
La/Py	13 0 ± 0.4	24.0 ± 0.7*
C AMP	1 46 ± 0.03	1 45 ± 0.04
Adenosine	0.4 ± 0.0	0 4 ± 0.1

– p < 0 05, – p < 0 01 – p < 0.001

In conclusion, results obtained in hypoxia demonstrate that adenosine concentrations cerebral cortex are elevated above normal only when the tissue hypoxia is sufficiently pr nounced to elevate AMP concentrations.

Status epilepticus

Tissue was frozen *in situ* either 10 s or 20 min following induction of status epilepticus wi bicuculline. In the first group the first spike discharges appeared in the EEG 7.5 ± 0 6 s fo lowing injection of bicuculline, and freezing was thus started about 2–3 s after the beginni of seizure discharge. In the second group animals showed a continuous seizure dischar during the entire 20 min period. Mean arterial blood pressure ranged from 140–170 mmHg i

TABLE IV The influence of 30 min of hypoxia with restricted hyperemia (hypoxia combined with unilater carotid artery occlusion) on the cortical energy stat as well as on the cortical concentrations adenosine and cyclic AMP in the ipsilateral hemisphere of 6 individual rats. The values (means ± S.E.) in the control group represents the concentrations in the ipsilateral hemisphere of 5 ra subjected to unilateral carotid artery occlusion t normoxia during 30 min. Adenylate energ charge was calculated as [ATP] + 0.5[ADP]/[ATP] + [ADP] + [AMP].

	Pa_{O_2} mmHg	PCr	ATP	ADP	AMP	E.C.	Adenosine	C-AMP
		mmol kg^{-1}					μmol kg^{-1}	
Controls (n = 5)	119 ± 6	4 47 ± 0.10	3.06 ± 0.05	0.299 ± 0 007	0.035 ± 0 002	0 948 ± 0.002	0.9 ± 0.1	1 61 ± 0 09
Hypoxia (individual values)	22.8	2.63	3 04	0.369	0.053	0.931	0 7	1 72
	19 4	3.95	3.08	0.322	0 041	0.941	1.0	1 47
	21 6	0.93	2.41	0 809	0 273	0.806	16.6	1.64
	22.1	0.68	1.80	1 12	0 362	0.719	104	4.50
	21.0	1 62	1 42	1.36	0.801	0.586	107	2.59
	24.9	0 19	0.52	1.55	1 40	0.373	140	2.40

TABLE V Cortical concentrations of phosphocreatine (PCr) adenine nucleotides (ATP ADP AMP), lactate, pyruvate (mmol kg^{-1}), the calculated adenylate energy charge (E.C.), the sum of the adenine nucleotides (Ad), and the lactate/pyruvate ratio, as well as concentrations of cyclic AMP and adenosine (μmol kg^{-1}) in rats subjected to seizures induced by bicuculline. The brains were frozen in situ with liquid nitrogen 2–3 after the first spike discharges appeared in the EEG (see text) or after 20 min period of continuous seizure activity

	Control 10	Time after bicuculline inj. 10 −4	20 min −6
PCr	4 54±0 06	2.60±0 07***	3 07±0 06
ATP	2.98±0 02	2.79±0.03***	2.76±0.01***
ADP	0.275±0 012	0.317±0 007	0.279±0 004
AMP	0 040±0 001	0 047±0.003*	0 045±0 001
E.C.	0 946±0 002	0 935±0 001**	0 940±0 001
ΣAd	3.30±0.02	3.16±0 02**	3 09±0.01***
La	1 45±0 05	3.33±0 04	11 6±0 07*
Py	0.126±0.006	0.240±0.017***	0.261±0 006
La/Py	13.2±0 4	14.7±0.9	44 4±0 9*
C AMP	1.51±0.05	2 80±0.19**	2.25±0 17*
Adenosine	0.8±0.1	0 8±0.1	0.7±0 0

p 0 05, p 0 01, *** $p<0.001$

control as well as in seizure groups and arterial P_{O_2} was kept above 100 mmHg. In the control group (n 10) arterial P_{CO_2} was 41 1 ±0.7 mmHg, and pH was 7.39 ±0.1 The values fell to 23.3 1 1 mmHg and 7 18 _0.02, respectively after 20 min of seizure activity (n − 6).

Table V shows that there was a fall in phosphocreatine (PCr) and ATP with small reductions in adenylate energy charge and adenine nucleotide pool in both seizure groups. After 20 min of seizure activity there was a marked increase in tissue lactate concentration, as well as of the lactate/pyruvate ratio. In both groups there were highly significant increases in cyclic AMP but unchanged adenosine concentrations.

During the seizure periods a very small, but statistically significant increase in AMP concentration (between 0 005 and 0 007 mmol kg^{-1}) was found, which corroborate the results reported by Chapman *et al* (1977). This very small increase in AMP did not influence the measured adenosine levels, indicating that AMP is a more sensitive indicator of changes in the phosphorylation state than adenosine. According to the regression line (adenosine (μmol kg^{-1}) 0 06 AMP (μmol kg^{-1}) − 1 7) describing the relationship between AMP and adenosine concentrations in shortlasting ischemia (Nordström *et al* 1977), the increase in AMP concentrations in the seizure groups, was probably too small to measurably affect the adenosine concentration.

Discussion

The present study has given three main results. First, the maximal rate of adenosine formation in ischemia is about 1 μmol kg^{-} s which is somewhat higher than the rate reported by Deuticke *et al.* (1966). Second, results obtained in hypercapnia give no indication that increased tissue oxygen tensions reduce adenosine concentrations below normocapnic control values. Third, neither hypoxia nor epileptic seizures are accompanied by elevated adenosine

TABLE III. Concentrations of phosphocreatine (PCr), adenine nucleotides (ATP ADP AMP), lactate, pyruvate (mmol kg^{-1}), the calculated adenylate energy charge (E.C.) and lactate/pyruvate ratio, as well as the concentrations of cyclic AMP and adenosine (µmol kg^{-1}) in rat cerebral cortex after 2 min of pronounced arterial hypoxia (Pa_{O_2} ≈ 20 mmHg).

	Control (n = 6)	Pronounced hypoxia 2 min (n = 6)
Pa_{O_2}	116±3 8	19.0±0.3
PCr	4 42±0.06	3.39±0.05
ATP	3 00±0.02	3 00±0.01
ADP	0.285±0.004	0.305±0.002*
AMP	0.049±0.001	0.047±0.001
E.C.	0.943±0.001	0.940±0.000*
Lactate	1 60±0.07	5 40±0.20*
Pyruvate	0.123±0.004	0.225±0.007*
La/Py	13.0±0 4	24 0±0.7*
C-AMP	1.46±0 03	1 45±0.04
Adenosine	0.4±0.0	0.4±0.1

* p<0 05 ** p<0.01 *** p<0.001

In conclusion results obtained in hypoxia demonstrate that adenosine concentrations in cerebral cortex are elevated above normal only when the tissue hypoxia is sufficiently pronounced to elevate AMP concentrations.

Status epilepticus

Tissue was frozen *in situ* either 10 s or 20 min following induction of status epilepticus with bicuculline. In the first group, the first spike discharges appeared in the EEG 7.5±0 6 s following injection of bicuculline, and freezing was thus started about 2–3 s after the beginning of seizure discharge. In the second group, animals showed a continuous seizure discharge during the entire 20 min period. Mean arterial blood pressure ranged from 140–170 mmHg in

TABLE IV The influence of 30 min of hypoxia with restricted hyperemia (hypoxia combined with unilateral carotid artery occlusion) on the cortical energy state, as well as on the cortical concentrations of adenosine and cyclic AMP in the ipsilateral hemisphere of 6 individual rats. The values (means ± S.E.) in the control group represents the concentrations in the ipsilateral hemisphere of 5 rats subjected to unilateral carotid artery occlusion at normoxia during 30 min. Adenylate energy charge was calculated as [ATP] + 0.5[ADP]/[ATP] + [ADP] + [AMP]

	Pa_{O_2} mmHg	PCr	ATP	ADP	AMP	E C.	Adenosine	C-AMP
		mmol kg^{-}					µmol kg^{-}	
Controls (n = 5)	119 ±6	4.47 ±0.10	3.06 ±0.05	0.299 ±0.007	0 035 ±0 002	0.948 ±0.002	0.9 ±0.1	1.61 ±0.09
Hypoxia (individual values)	22.8	2.63	3.04	0.369	0.053	0.931	0.7	1 72
	19 4	3.95	3.08	0.322	0 041	0.941	1.0	1 47
	21.6	0.93	2.41	0.809	0.273	0.806	16.6	1 64
	22.1	0.68	1.80	1 12	0.362	0.719	104	4.50
	21.0	1 62	1 42	1.36	0.801	0 586	107	2.59
	24.9	0.19	0.52	1.35	1 40	0.373	140	2.40

hypothesis they do not exclude the possibility that adenosine is an important regulator of cerebrovascular resistance. Thus, since the true intra- and extracellular concentrations are close to the limits of detection moderate increases in concentration, that may well have vascular effects, may be difficult to detect. Furthermore, if adenosine is released at extracellular sites by mechanisms akin to those postulated for purinergic nerves (see Burnstock 1975), and if adenosine is removed by cellular uptake mechanisms, overall tissue concentrations might not rise.

In summary we conclude that if adenosine mediates vasodilatation in the brain it must do so via more subtle mechanisms than those involving increased breakdown of AMP triggered by an imbalance between energy conserving and energy utilization pathways, with a consequent accumulation of adenosine in the brain.

Relationship between adenosine and cyclic AMP Our previous results demonstrated a linear relationship between tissue concentrations of adenosine and cyclic AMP during short-lasting ischemia (Nordström *et al.* 1977). In the present work we found no increase in the tissue levels of cyclic AMP (or adenosine) in hypoxia, if the increase in CBF was sufficient to provide the tissue with oxygen to keep a normal energy balance and prevent a deterioration of the phosphorylation state of the adenine nucleotides. Only if hypoxia was exaggerated with unilateral carotid artery occlusion (hypoxia with restricted hyperemia) was there an increase in cyclic AMP concentration, provided that the tissue hypoxia had been severe enough to cause a derangement of the phosphorylation state with pronounced accumulation of adenosine. Thus, in situations with marked disturbances of the cerebral energy state, adenosine accumulation may well trigger an increase in cyclic AMP concentration.

Earlier investigations have demonstrated an increase in cyclic AMP during neuronal hyperactivity This has been observed in electrically stimulated brain slices (Kakiuchi *et al* 1969) as well as *in vivo* after pharmacologically or electrically induced seizures (Sattin 1971) Furthermore, *in vitro* experiments have demonstrated increased concentration of cyclic AMP in perfused brain slices after incubation with adenosine (Sattin and Rall 1970 Schultz and Daly 1973). However the present results with bicuculline-induced seizures give evidence of a highly significant increase in cyclic AMP without any change in adenosine concentration (or substantial change in phosphorylation state) We thus conclude that other mechanisms than adenosine accumulation seem to be responsible for the increase in tissue concentration of cyclic AMP during neuronal hyperactivity

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concentrations unless there is accumulation of AMP Since the results are at variance with data published by others we will discuss these results, and put some emphasis on the possible involvement of adenosine in the control of CBF as well as on its relationship to cyclic AMP concentration. However it seems appropriate to begin by considering tissue adenosine concentration under resting control conditions.

Cortical adenosine concentration under control conditions With conventional chromatographic techniques, adenosine concentrations in cerebral tissues are below the limit of detection (*e g* Deuticke *et al* 1966) More sensitive techniques were used by Kleihues *et al* (1974) in cats, by Berne *et al* (1974) in dogs, and by Rubio *et al* (1975) in rats who obtained values of about 20 10 and 5 μmol kg^{-1} respectively However in these studies the tissue was removed with a spatula or a bone rongeur inducing shortlasting ischemia before freezing occurred Our previous results (Nordström *et al* 1977) as well as the present ones, have shown that if the tissue is frozen through the exposed dura or the exposed bone without prior interruption of the circulation, adenosine concentrations do not exceed 1 μmol kg^{-1} Our previous results also showed that, at least in rats, the moderate trauma induced by a craniotomy may lead to markedly elevated adenosine concentrations, and values of 1 μmol kg^{-1} or lower are obtained only if special precautions are taken not to damage the tissue during the craniotomy (see Nordström *et al* 1977) We conclude that measurements of tissue adenosine concentrations require optimal freezing conditions. Since the present technique gives high values for PCr and ATP and low ones for ADP and AMP we conclude that the freezing conditions should be relatively optimal for adenosine as well.

Relationship between tissue P_{O_2} and adenosine concentration Results reported by Rubio *et al* (1975) suggested that there was an inverse relationship between tissue P_{O_2} and adenosine concentration since the latter was reduced in hypercapnia and increased in moderate hypoxia (10 O) Our data fail to corroborate these results since adenosine concentrations were unaltered in hypercapnia and in hypoxia, whether moderate or severe. In these situations, tissue AMP concentrations were unaltered and the unchanged adenosine concentrations thus supports the assumption that the 5 -nucleotidase reaction is substrate-dependent (Kluge *et al* 1972). Additional support for this view was obtained by results in hypoxic animals in which the tissue hypoxia was exaggerated by unilateral carotid artery ligation (*cf* Salford *et al.* 1973 Salford and Siesjö 1974) Thus, adenosine concentrations rose only when hypoxia was sufficiently severe to raise AMP concentrations above normal

Coupling of adenosine concentration and CBF Using the same experimental conditions we have previously measured CBF in hypoxia and status epilepticus, and these measurements have been correlated to tissue concentrations of labile organic phosphates (for literature, see Jóhannsson and Siesjö 1975 Siesjö *et al* 1975 Nilsson *et al* 1977). These results demonstrate that the present degrees of hypoxia increase CBF about 5-fold without altering tissue AMP concentrations. In status epilepticus, CBF increases by similar degrees, and there is either a very small, or no increase in AMP concentration (Meldrum and Nilsson 1976, Chapman *et al* 1977) Obviously the data give no support to the hypothesis that vasodilatation in hypoxia or during epileptic seizures is caused by accumulation of adenosine in the tissue.

It should be explicitly stated that although the present results fail to support the adenosine

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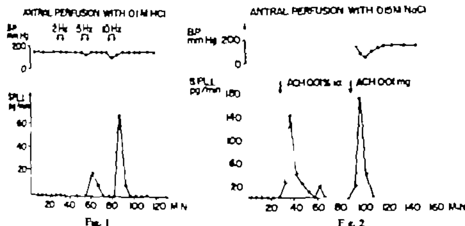


Fig. 1

Fig. 2

Fig. 1. Release of SPLI into the antral lumen following electrical vagal stimulation with 2, 5 and 10 Hz. In this experiment no release was induced by vagal stimulation with 2 Hz, whereas stimulation with 5 and 10 Hz did cause such a release. In this experiment the antrum was perfused with 0.1 M HCl.

Fig. 2. Release of SPLI into the antral lumen following perfusion of the antrum with 0.01 % Ach solution and following injection of 1 mg Ach intravenously. The antrum was perfused with 0.15 M NaCl.

ten periods in tubes surrounded by ice. Acid samples were neutralized with 0.01 M NaOH, using phenolphthalein as an indicator, then all samples were boiled for 10 min and stored at −20°C until assayed for SPLI-content by radioimmunoassay. The details of this assay have been given in previous papers (Nilsson et al. 1975, Nilsson et al. 1977). The antibodies used (K 25) are directed against the whole SP molecule. Removal of one single aminoacid from the SP molecule virtually abolishes all immunoreactivity with this antibody (T[illegible] be publ.)

The vagi were isolated and ligated in the cervical region. The distal parts of the nerves were placed on electrodes which were connected to a Grass stimulator (S [illegible]). Stimulations were performed at the following parameters: [illegible] V, 2 [illegible] duration and 2–10 Hz.

To test the effect of acetylcholine (Ach), this substance was added to the perfusion medium (HCl and NaCl) in concentrations ranging from 0.01–1 [illegible] and the antrum was perfused for 10 min. In 2 experiments 0.01 mg Ach was injected i.[illegible] Adrenaline (0.01 mg) was also injected in 3 experiments.

The release rate of SPLI (pg/min) was calculated by multiplying SPLI concentrations and the perfusion rate. In order to obtain the amounts of SPLI released by the different stimulatory agents, basal release was subtracted from the total output of SPLI observed after stimulation.

Results

SPLI was found in antral perfusates in 8 of 12 investigated cats. The basal release varied between 0 and 60 pg/min. pH of the perfusion medium did not seem to influence the release rate.

Electrical vagal stimulation caused a release of SPLI into the antrum (Fig. 1). Release responses (100–800 pg) were obtained within a frequency range from 2–10 Hz. The output varied between 100–800 pg during perfusion with NaCl and between 170–550 pg during perfusion with HCl (Table I).

Antral perfusion with Ach (0.01–1 %) caused a release of SPLI (400–1 700 pg) as did i.v. injections (0.01 mg) (Table II, Fig. 2). Adrenaline (0.01 mg) i.v. released SPLI as well (Fig. 3, Table II).

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Release of substance P like immunoreactivity into the antral lumen of cats

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Abstract

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SPLI can be detected in a tral perfusates of cats. Experiments were performed in anesthetized cats provided with antral pouches. The pouches were perfused with 0.1 M HCl nd 0.15 M NaCl. The concentration of SPLI in the perfusates was determined by radioimmunoassay Basal output varied bet een 0-60 pg/min. Electrical vagal stimulation (2 10 Hz) caused release responses varying between 100-800 pg. Ach given i.v or intraantrally also released SPLI into the antral lumen as well as adrenaline given i.

The central nervous system and the intestine, wherefrom substance P (SP) was originally extracted (Euler and Gaddum 1931) constitute the two quantitatively most important locations of SP in the mammalian body (Pernow 1953) In the gastrointestinal tract substance P-like immunoreactivity (SPLI) has been demonstrated in nerves as well as in endocrine like cells (Nilsson *et al* 1975 Pearse and Polak 1975)

Other gastrointestinal peptides are released into the gastrointestinal lumen in parallel to being secreted into the blood stream Thus gastrin, somatostatin and VIP have been demonstrated in antral and/or duodenal perfusates following electrical vagal stimulation (Uvnäs-Wallensten and Rehfeld 1976 Uvnäs-Wallensten *et al* 1977 Uvnäs-Wallensten 1978, Uvnäs-Wallensten *et al* 1978 a, b). We therefore looked for SPLI in antral perfusates.

Methods

The experiments were performed on 12 cats (2-4 kg) fasted for 18 h and anesthetized with chloralose-urethane (50 and 100 mg/kg L .). The blood pressure was recorded through a catheter inserted into one of the femoral arteries. The rectal temperature was recorded and kept t 38-39°C by heati g the animal ith an electrical heating pad. In all cats an acute antral pouch was created by tying ligatures around the pylorus and the antrum-corpus boundary care being taken to avoid damage to nerves and vessels. The pouch was perfused with 0.15 M NaCl or 0.1 M HCl, introduced via a pyloric catheter at rat of 1.0 ml/min. The perfusate left the antrum through a cannula inserted into the antral wall and was collected during 5 or 10

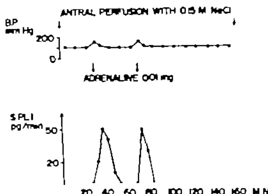


Fig. 3 Release of SPLI into the antral lumen following injections of adrenaline (0.01 mg) i.v. The antrum was perfused with 0.15 M NaCl

vasodilator actions (Euler and Gaddum 1931 Pernow 1953 Hedqvist and Euler 1975). The present results show that antral SPLI can be released by nervous activation (electrical vagal stimulation) as well as by humoral agents (Ach and adrenaline). Whether the SPLI release caused by these mechanisms are of any physiological importance cannot be stated at present Other gastrointestinal peptides such as gastrin, somatostatin and VIP can also be released by electrical vagal stimulation within the same frequency range (2–10 Hz) (Uvnäs-Wallensten *et al.* 1976, Uvnäs-Wallensten *et al.* 1977 Schaffalitzky de Muckadell *et al.* 1977). This similarity indicates that the release of SPLI caused by vagal stimulation may occur physiologically just as the well known vagally induced gastrin release.

In conclusion we have shown that SPLI occurs in antral perfusates of cats. We have also been able to induce a release of SPLI by electrical vagal stimulation, by Ach given intraluminally or intravenously and also by adrenaline given i.v

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TABLE I Release of SPLI into the antral lumen of cats induced by electrical vagal stimulation (2 10 Hz) during antral perfusion with 0.15 M NaCl and 0 1 M HCl.

Exp no	Hz	SPLI pg 0.15 M NaCl	SPLI pg 0.1 M HCl
1	2		0
2	5		170
3	10		550
4	10		210
5	2	100	
6	5	255	
7	5	800	
8	5	180	
9	10	190	

Discussion

The present results thus show that SPLI like gastrin, somatostatin and VIP is released into the antral lumen of cats. An increased output of SPLI could be induced by electrical vagal stimulation, by Ach given i v or into the antrum as well as by adrenaline given i.v

Recent in vitro studies have shown SPLI to be released from nervous tissue in response to electrical stimulation and to elevation of potassium levels in the surrounding medium (Otsuka *et al* 1976, Iversen *et al* 1976)

SPLI occurs in nervous structures as well as in endocrine cells within the gastrointestinal tract No SPLI containing cells have been observed proximal to the pylorus. If so the released peptide must be assumed to diffuse through the mucosal layer to reach the lumen

The physiological function of SP in the gastrointestinal tract is still unsolved. SP has by several authors been given a hypothetical role as a regulator of gut motility but SP also has

TABLE II Release of SPLI (pg) induced by 0.01 0.1 and 1% solutions of Ach administered via the perfusate and by intravenous injections of Ach (0.01 mg) and 0.01 mg of adrenaline given i v The release of SPLI was calculated as concentration of SPLI in perfusates times flow rate (1 ml/ml) The output caused by a stimulation was calculated by subtracting basal release of SPLI from total amounts observed after the stimulation.

Exp. no.	Ach %	SPLI pg 0.15 M NaCl	SPLI pg 0.1 M HCl
1	0 01		938
2	0.1		650
3	1		1 700
4	1	1 100	
5	0.01 mg (i.v)	850	
6			400
	Adrenaline mg		
1	0.01	500	
2	0.01	400	
3	0.01		100

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Inhibition of acetylcholine release in guinea pig ileum by adenosine

By

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Abstract

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The effect of adenosine on cholinergic neuroeffector transmission was studied in the isolated guinea pig ileum. Adenosine caused a dose-dependent and inverse frequency-dependent inhibition of contraction responses to transmural nerve stimulation. Blockade of adrenergic neurotransmission did not alter the inhibitory effect of adenosine. Adenosine also inhibited contraction responses to serotonin, angiotensin and high potassium, but not the responses to acetylcholine, histamine or direct electrical stimulation of the smooth muscle cells. Adenosine had little effect on basal outflow of acetylcholine but inhibited markedly and reversibly the release of acetylcholine induced by nerve stimulation. Acetylcholine was determined with gas chromatography-mass spectrometry. The results provide direct evidence that adenosine inhibits cholinergic neuroeffector transmission in the gut by a prejunctional action on acetylcholine release. This may be of functional importance since adenine compounds are released during stimulation of intestinal nerves.

Key words: Acetylcholine, adenosine, choline, guinea pig ileum, gas chromatography-mass spectrometry, neuroeffector transmission, smooth muscle, transmitter release

Modulation of transmitter release from nerve endings by substances released from nerves (e.g. the transmitter itself) or from the effector organ has been demonstrated especially as regards sympathetic nerves (*cf.* Starke 1977, Westfall 1977). Recently adenosine was proposed as a possible modulator of adrenergic neuroeffector transmission in the kidney, adipose tissue and vas deferens (Hedqvist and Fredholm 1976). There is evidence in the literature that adenosine (and other purine compounds) also affect acetylcholine (ACh) release from motor nerve endings (Ginsborg and Hirst 1972, Ribeiro and Walker 1975) as well as from cholinergic nerves in the gut (Sawynok and Jhamandas 1976, Vizi and Knoll 1976, Leighton and Parmeter 1977). It therefore seemed of interest to further study if adenosine could interact with cholinergic autonomic neurotransmission, and to study this in a system where it would be possible to directly measure release of the putative neurotransmitter substance. The plexus-containing longitudinal muscle preparation of guinea pig ileum has offered a useful system to elucidate the adrenergic interaction with ACh release (Paton and Vizi 1969, Kosterlitz *et al.* 1970, Wikberg 1977) and was therefore chosen for the present study on adenosine interaction with cholinergic nerves. A preliminary account of the present results has been presented elsewhere (Gustafsson *et al.* 1977).

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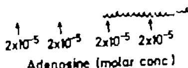
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Naloxone 10^{-4}M

Fig. 1. Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. Wash at dots. Naloxone added to bath fluid during the last half of experiment as indicated by horizontal bar



Results

The isolated longitudinal muscle of the guinea pig ileum contracts in response to transmural stimulation, given as single pulses or trains of pulses of 1 ms duration. Since the response is abolished by atropine (3×10^{-7} M) or tetrodotoxin ($3\text{–}6 \times 10^{-7}$ M) it is probably due to activation of cholinergic nerve fibres. The contraction response to this type of stimulation was inhibited by adenosine administered over a wide range of concentrations. The inhibition was manifest after 10–20 seconds, usually reached its maximum within 1–2 minutes and was readily reversed upon washing (Fig. 1). The inhibitory effect of adenosine was essentially

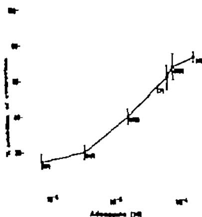


Fig. 2



Fig. 3

Fig. 2. Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. Vertical axis: The depression by adenosine of contraction responses expressed as percent of the response prior to the administration of drug. Horizontal axis: Log molar concentration of adenosine. Mean values, number of experiments in brackets. Vertical bars indicate ± S.E.

Fig. 3. Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (3 Hz, 5-10 pulses, 1 ms, supramaximal voltage) 9 min intervals. Wash at dot.

Methods

General procedure

Male guinea pigs (300–500 g) were stunned and bled. The distal part of the ileum was isolated and was used for two types of preparations (I) plexus-containing longitudinal muscle according to Paton and Vizi (1969), and (II) whole segment of ileum with intact perivascular nerves as described by Finkleman (1930). The preparation was mounted in organ baths containing Tyrode's solution of the following composition (concentrations in mM): Na 149, K 2.7, Ca 1.8, Mg 0.5, Cl 144, HCO_3 11.9, H_2PO_4 0.4, glucose 5.5. The Tyrode was kept at 37°C and was continuously bubbled with 5% CO_2 in O_2.

Effector responses in longitudinal muscle

The preparation (20–100 mg) was mounted in a 5 ml bath. Muscle tone and contractions were recorded isotonically (load 0.2–0.5 g) using a smooth muscle transducer (Harvard Apparatus 343) and an ordinate writer (Honeywell Electronik 194). Biphasic single pulses or trains of pulses (duration 1 ms, supramaximal voltage), specifically activating the nerves, were delivered by means of a pair of platinum electrodes along the walls of the bath, and a Grass S88 stimulator. In experiments where tetrodotoxin was used the bath volume was 125 ml and the electrodes were placed 5 mm from and parallel to the preparation. Direct stimulation of the smooth muscle cells was accomplished with trains of square wave pulses (duration 20–30 ms, supramaximal voltage).

Release of ACh

Strips of plexus-containing longitudinal muscle (80–130 mg) were mounted in a 1 ml organ bath having platinum electrodes along the walls. The preparation was allowed to stabilize over a period of two hours of intermittent stimulation. During the release experiments the Tyrode contained eserine (3 µM) and was changed every 5 min. The preparation was electrically stimulated with biphasic pulses (0.5–3 Hz, 1 ms, supramaximal voltage) for 280 s at 20 min intervals. Muscle contractions were recorded isometrically by means of a Grass FT03 strain gauge transducer and a Grass polygraph. Collected bath fluid was immediately chilled, acidified to pH 6.5 and stored at −70°C until analyzed for ACh and choline (Ch) according to the method of Karlén *et al.* (1974). Briefly, deuterated (D_4) ACh and Ch were added as internal standards, whereafter extraction was performed with dipicrylamine. After demethylation with sodium benzenethiolate the samples were extracted and run on an LKB gas chromatograph-mass spectrometer. ACh and Ch levels were calculated from peak height ratios between ions of deuterated and non-deuterated compounds. Accurate corrections were made for recovery from control samples containing known amounts of ACh and Ch. Apart from estimation by measurement of fragments m/e 58 and 64 the presence of fragments m/e 71 and 77 was confirmed as evidence for measurement of Ch derivatives.

Effector responses in whole segments of ileum

Segments of ileum (2–3 cm) with adjacent mesentery were mounted in a 50 ml bath and arranged for both transmural and periarterial nerve stimulation. Contractions were elicited through electrodes parallel to and 5 mm apart from the preparation, and by a Grass S88 stimulator delivering a single biphasic pulse (1 ms, supramaximal voltage) each minute. The adrenergic nerves were electrically stimulated (3–10 Hz, 1 ms, 5–10 V) through periarterial electrodes at appropriate intervals. Muscular activity was recorded isotonically as described above.

Drugs

Acetylcholine hydrochloride (Sigma Co., St Louis, USA), adenosine (Sigma), angiotensin (Hypertensin, Ciba-Geigy AG, Basle, Switzerland), atropine sulfate (Sigma), eserine sulfate (Sigma), guanethidine sulfate (Ciba-Geigy), histamine hydrochloride (ACO, Stockholm, Sweden), 5-hydroxytryptamine creatinine sulfate (serotonin, Sigma), naloxone hydrochloride (Endo Labs, Garden City, N.Y., USA), phentolamine hydrochloride (Ciba-Geigy), tetrodotoxin (Sigma). D_4-ACh and D_4-Ch were synthetized at the Department of Toxicology, Karolinska Institutet. All chemicals used were of reagent grade. Concentrations refer to the base unless otherwise stated.

Statistics

Statistical significance was tested according to Student's t-test for paired and unpaired variates.

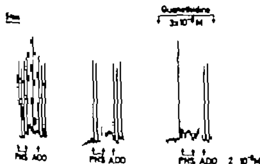


Fig. 6

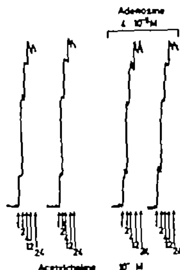


Fig. 7

Fig. 6. Isolated whole segment of the guinea pig ileum with adjacent mesentery. Contraction responses to transmural stimulation (single pulses, 1 ms, supramaximal voltage) at 1 min intervals. PNS = stimulation of mesenteric periarterial nerves (10 Hz, 1 800 pulses, 1 ms, 5 V). ADO = adenosine 2 10^{-4} M added to bath fluid. Washing between registration periods. Constant magnification of responses throughout the expt. Guanethidine added to bath fluid as indicated by horizontal bar

Fig. 7. Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses. Acetylcholine added to bath fluid in cumulative fashion giving total concentration as indicated at arrows. Washing between registrations. Constant magnification of responses throughout the expt. Adenosine added to bath fluid as indicated by horizontal bar

tion on contraction responses to transmural nerve stimulation. The inhibitory action of adenosine on responses to transmural stimulation was still present, however (Fig. 6).

Contraction responses of the longitudinal muscle were also obtained by increasing the potassium concentration of the bath fluid to 20 mM through addition of KCl. These responses were, when sensitive to atropine (3 10⁻ M), inhibited by adenosine. Adenosine, in concentrations inhibiting responses to transmural nerve stimulation, did not alter contraction responses to exogenous ACh, which was administered in submaximal concentrations or cumulatively to give a complete dose-response curve (Fig. 7). After administration of tetrodotoxin (3–6 10⁻ M), which blocked the contraction responses to transmural nerve stimulation, direct stimulation of the smooth muscle cells was obtained by prolonging the pulse duration to 20–30 ms. The ensuing contraction responses (uninfluenced by atropine 0.3–1 10^{-6} M and guanethidine 3 10^{-6} M) were not altered by adenosine in concentrations causing at least 50 inhibition of neurogenic responses in the same preparation (4 expts.) (Fig. 8). Contraction responses to histamine were also uninfluenced by adenosine. On the other hand, serotonin (10^{-6} M) and angiotensin II (5 10^{-6} M) produced contraction responses, which were inhibited by adenosine in concentrations depressing responses to transmural nerve stimulation (Fig. 9)

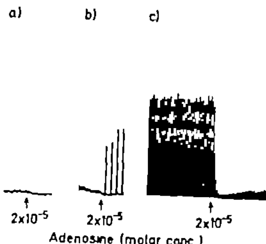


Fig. 4 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (1 ms, supramaximal voltage) at varied frequencies and intervals. a) 3 Hz, 15 pulses at 1 min intervals. b) Single pulses at 1 min intervals. c) Single pulses at 10 s intervals. Constant magnification of responses throughout the expt. Wash at dots.

unaffected by naloxone, suggesting that it was not due to activation of opiate receptors (Fig. 1). Although there was some variation in reactivity between different preparations, a given dose usually caused very reproducible responses throughout an experiment. At a stimulation frequency of 3 Hz a significant inhibition of the contraction response was obtained with adenosine, 7 10 M and the inhibition progressively increased with increasing doses of adenosine, approaching 80% at 2 10^{-4} M (Fig. 2). Adenosine caused inhibition of both phasic ("twitch") and tonic contractions induced by transmural nerve stimulation (Fig. 3) Adenosine was more prone to inhibit the contraction response when the preparation was stimulated with single shocks each minute, and even more so when pulses were delivered continuously at 0 1 Hz (Fig 4) At 1 pulse per minute 53 ± 5% (mean ± S.E., n 9) inhibition was obtained by adenosine 2 10^{-4} M and at 0 1 Hz it was 84 ± 4% (n 8). The difference between these two values is highly significant (p 0 001) A common phenomenon was a tendency of adenosine-inhibited responses to slowly return towards the control level in spite the preparation had not been washed In a few cases this was very prominent (Fig. 5), and repeated doses could be given, still eliciting comparable inhibition without interposed washing. This suggests elimination of the drug from its site of action or some other type of reactivation process.

Administration of guanethidine (3 10^{-4} M) or phentolamine (3 10^{-4} M) did not alter the inhibitory action of adenosine on contraction responses to transmural nerve stimulation. To ascertain a complete block of adrenergic neurotransmission, experiments were performed on whole segments of ileum prepared according to Finkleman (1930). Guanethidine (3 10^{-4} M) abolished or even reversed the inhibitory effect of perivascular nerve stimula

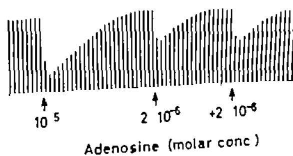


Fig 5 Isolated lo gitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses t transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min tervals. Preparation not washed during the expt.

TABLE I Release of acetylcholine and choline from the exercised longitudinal muscle of the guinea pig ileum, as determined by gas chromatography-mass spectrometry. Bath fluid was collected at 5 min intervals and transmural stimulation delivered for 280 s at 0.5 or 3 Hz every 20 min. Rest denotes the release during the 5 min period just prior to stimulation. Values in pmol g^{-1} min^{-1}. Mean and S.E. of 6 expts. Statistical significance tested according to Student's t-test for paired data (** p 0.01)

	Control		Adenosine 2 10^{-4} M	
	ACh	Ch	ACh	Ch
0.5 Hz				
Rest	83±6	757±99	68±5	666±82
Stim	190±24	797±77	97±9**	681±76
3 Hz				
Rest	130 37	910±132	98±22	819±167
Stim	401±77	962±179	378±44**	899±167

at the 0.1% level according to Student's t-test. After washout of adenosine the overflow of ACh promptly returned to the control level (Table II).

The release of Ch was not significantly increased by transmural stimulations, and not altered by administration of adenosine. Ch release was higher (7 9 times) than ACh release during resting periods, and also during stimulations (1.5–7 times) (Table I)

Discussion

The present study has confirmed previous suggestions that adenosine inhibits ACh release from the guinea pig ileum (see Introduction). We have presented both indirect evidence and direct proof by chemical determination of ACh with gas chromatography-mass spectrometry. It was originally shown by Takagi and Takayanagi (1972) that different purine compounds, namely ATP cyclic AMP and dibutyryl cyclic AMP inhibited contractions of the guinea pig ileum induced by electrical stimulation, serotonin and nicotine, while those induced by ACh were unaffected. Furthermore, ACh release (estimated by bioassay) following electrical stimulation was depressed during nucleotide administration. Later Ginsborg and Vizzacchio (1975) showed that the nucleoside adenosine was also able to inhibit electrically induced contractions of the guinea pig ileum, which has been concluded to be due to in-

TABLE II Net overflow of acetylcholine from the exercised longitudinal muscle of the guinea pig ileum, evoked by consecutive periods of transmural nerve stimulation for 280 s at 20 min intervals. Acetylcholine determined by gas chromatography-mass spectrometry. Net overflow was calculated by subtraction of the acetylcholine released during the 5 min period prior to stimulation from the amount released during transmural stimulation. Percent of control, number of expts. in brackets. Statistical significance tested according to Student's t-test for paired data (*** = p 0.001)

	Stim 1 (Control = 100)	Stim 2 Adenosine 2 10^{-4} M	Stim 3
0.5 Hz	100 (6)	27±7 (6)***	90±13 (4)
3 Hz	100 (6)	39±3 (6)***	106±8 (4)

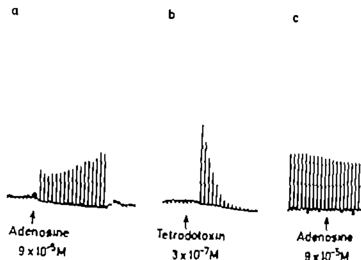


Fig. 8 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation before and during tetrodotoxin treatment. a) and b) Contraction responses to transmural stimulation with pulses of short duration (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. c) Direct smooth muscle activation by pulses of long duration (10 Hz, 50 pulses, 20 ms, supramaximal voltage) at 1 min intervals (magnification of contraction responses × 2). Wash at dot.

Before eserinization overflow of ACh in response to nerve stimulation could not be detected. In the presence of eserine (3×10^{-6} M), however, nerve stimulation gave detectable overflow of ACh, which after 1 hour had a S_1 to S ratio of 1.03 ± 0.11 (n = 8) at 0.5 Hz and 1.00 ± 0.06 (n = 6) at 3 Hz. The outflow of choline was little influenced by nerve stimulations and slowly decreased throughout the experiments. Adenosine (2×10^{-4} M) had no discernible effect on spontaneous outflow of ACh but significantly inhibited the overflow of ACh in response to transmural stimulation. The efflux of Ch was not significantly affected by this adenosine concentration (Table I). Adenosine (2×10^{-4} M) inhibited the net overflow of ACh in response to nerve stimulation (overflow during nerve stimulation minus basal overflow) by 41 % at 3 Hz and 73 at 0.5 Hz stimulation. This difference is significant

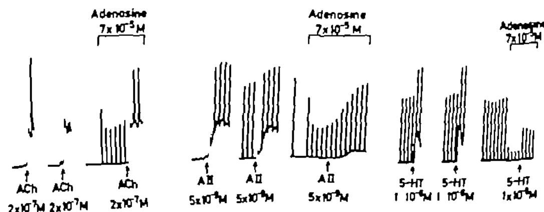


Fig. 9 Isolated longitudinal muscle of the guinea pig ileum. Effect of adenosine on contraction responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min interval and on contraction responses to added agonists. ACh = acetylcholine, A II = angiotensin II, 5-HT = serotonin. Wash at dots. Adenosine added to bath fluid as indicated by horizontal bars.

Table I. Release of acetylcholine and choline from the stimulated longitudinal muscle of the guinea pig ileum, as determined by gas chromatography-mass spectrometry. Bath fluid was collected at 5 min intervals and transmural stimulation delivered for 2[illegible] at 0.5 or 3 Hz every 20 min. Rest denotes the release during 5 min period just prior to stimulation. Values in pmol g^{-1} min^{-1}. Mean and S.E. of 6 expts. Statistical significance tested according to Student's t-test for paired data (** p [illegible] 0.01)

	Control		Adenosine 2 10^{-4} M	
	ACh	Ch	ACh	Ch
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3 Hz				
Rest	138 + 37	910 ± 132	98 ± 22	819 ± 167
Stim	[illegible]01 77	962 ± 179	378 ± 44**	899 ± 167

[illegible] level according to Student's t-test. After washout of adenosine the overflow of ACh promptly returned to the control level (Table II).

The release of Ch was not significantly increased by transmural stimulations, and not altered by administration of adenosine. Ch release was higher (7–9 times) than ACh release during resting periods, and also during stimulations (1.5–7 times) (Table I).

Discussion

The present study has confirmed previous suggestions that adenosine inhibits ACh release from the guinea pig ileum (see Introduction). We have presented both indirect evidence and direct proof by chemical determination of ACh with gas chromatography-mass spectrometry. It was originally shown by Takagi and Takayanagi (1972) that different purine compounds, namely ATP, cyclic AMP and dibutyryl cyclic AMP, inhibited contractions of the guinea pig ileum induced by electrical stimulation, serotonin and nicotine, while those induced by ACh were unaffected. Furthermore, ACh release (estimated by bioassay) following electrical stimulation was depressed during nucleotide administration. Later Glitzler and [illegible] (1975) showed that the nucleoside adenosine was also able to inhibit electrically induced contractions of the guinea pig ileum, which has been concluded to be due to in-

Table II. Net overflow of acetylcholine from the stimulated longitudinal muscle of the guinea pig ileum, evoked by consecutive periods of transmural nerve stimulation for 2[illegible] at 20 min intervals. Acetylcholine determined by gas chromatography-mass spectrometry. Net overflow was calculated by subtraction of the acetylcholine released during the 5 min period prior to stimulation from the amount released during transmural stimulation. Percent of control, number of expts. in brackets. Statistical significance tested according to Student's t-test for paired data (** [illegible] p [illegible] 0.001)

	Stim 1 (Control 100 %)	Stim 2 Adenosine 2 10^{-4} M	Stim 3
0.5 Hz	100 (6)	27 ± 7 (6)*	90 ± 13 (4)
3 Hz	100 (6)	39 ± 3 (6)*	106 + 8 (4)

hibition of ACh release since responses to applied ACh were unaltered (Sawynok and Jhamandas 1976)

The inhibition by adenosine of contractile responses to nerve stimulation is not likely due to interactions with adrenergic mechanisms. The effect of adenosine is unaffected by phentolamine (Vizi and Knoll 1976 and present results) or by phenoxybenzamine (Sawynok and Jhamandas 1976) Furthermore our results demonstrate that guanethidine in a dose that completely eliminates the inhibitory effect of periarterial nerve stimulation (presumably adrenergic) does not affect the response to adenosine. Finally adenosine is known to inhibit the release of noradrenaline in a number of tissues (Hedqvist and Fredholm 1976, Verhaeg *et al* 1977). Theoretically inhibition of noradrenaline release in the guinea pig ileum would tend to, if anything, increase ACh release and contractile responses would be enhanced (Paton and Vizi 1969).

It is well known that morphine gives a similar type of inhibition of contractile responses as adenosine in the guinea pig ileum, presumably due to inhibition of ACh release (Paton 1957 Schaumann 1957) Indeed it has been suggested, referring to the actions of adenosine and morphine that "the biochemical pathways responsible for mediating the effects may share some common elements" (Gintzler and Musacchio 1975). Endogenous opioid peptides have profound actions in the guinea pig ileum (Waterfield *et al* 1977) and have been demonstrated to be released from the tissue by electrical stimulation (Schulz *et al* 1977). Therefore it is possible that the effect of adenosine could be mediated directly over morphine receptors or via the release of endogenous substances with morphine-like activity However neither we nor Sawynok and Jhamandas (1976) could inhibit the action of adenosine by the morphine receptor antagonist naloxone, rendering this possibility less likely On the other hand, the possibility that morphine might release an adenosine-like substance (Sawynok and Jhamandas 1976) must remain open.

In the rabbit small intestine adenosine, and related compounds, decrease tone and contractility by a mechanism apparently unrelated to nervous elements (Drury and Szent-Györgyi 1929 Euler and Gaddum 1931 McKenzie *et al* 1977 Ally and Nakatsu 1977) However in the guinea pig ileum the effect is in all probability due to an action on nerves Thus the effect of exogenous ACh is unaffected by adenosine (Sawynok and Jhamandas 1976 present results), as are the effects of the directly acting agonist histamine (present results) Concentrations of adenosine that are highly effective on transmural stimulation with pulses of short duration are without effect on responses to stimulation with pulses of long duration after tetrodotoxin (Fig. 8), which directly activates the smooth muscle elements Furthermore, the effects of several agonists supposed to act via release of ACh, *i.e.* potassium, serotonin (Rocha e Silva *et al* 1953 Gaddum *et al* 1957), angiotensin II (Robertson and Rubin 1962) and cholecystokinin (Vizi *et al* 1973), are inhibited by adenosine (Vizi and Knoll 1976, present results).

The best evidence for a prejunctional action of adenosine is the direct demonstration that it inhibited ACh release. Using bioassay (guinea pig ileum) Vizi and Knoll (1976) reported that 4 10^{-4} M adenosine inhibited ACh release induced by 0.2 Hz. In a recent abstract Leighton and Parmeter (1977) state that adenosine inhibits ^{14}C ACh release from ^{14}C-Ch labelled guinea pig ileum. In the present study we report that the release of chemically

identified endogenous ACh is inhibited by adenosine. The inhibition of ACh release by adenosine was frequency dependent. 2×10^{-4} M adenosine inhibited ACh release by about 75% at 0.5 Hz but only by 40% at 3 Hz. Although results obtained with bioassay and chemical determination cannot be directly compared (Gustafsson *et al.*, to be publ.) the results of Vizi and Knoll (1976) suggest an even larger effect of adenosine at 0.2 Hz. In this context it should be mentioned that the inhibitory effect of adenosine on contractions due to electrical stimulation was dependent on the mode of stimulation used. Thus contractions due to single pulses were inhibited more than those due to short bursts at 3 Hz for 5 s. Inhibition was further increased if the interval between single pulses was reduced from 1 min to 10 s. It has been suggested that adenosine influences transmembrane calcium fluxes (Schrader *et al.* 1977) and it has also been proposed that presynaptic regulation of neurotransmitter release is ultimately dependent on altered calcium transport (*cf.* Westfall 1977) which normally triggers transmitter release (*cf.* Hubbard 1970). However the biochemical mechanisms underlying the frequency dependent inhibition of ACh release by adenosine and the dependence on the type of stimulation given are so far obscure.

Adenine derivatives are released upon nerve stimulation in various parts of the gut (Burnstock *et al.* 1970, Satchell and Burnstock 1971, Su *et al.* 1971). Adenosine is a major component hereof (Satchell and Burnstock 1971). Therefore it is possible that the described effect of adenosine on ACh release may be physiologically relevant. This possibility is independent on whether proposed purinergic nerves (Burnstock 1972) exist or not since it does not require that the purine compound subserves a transmitter function. Adenosine (or a related compound) could modulate ACh release irrespective of whether it is of nervous or non-nervous origin.

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Studies on the melanin-affinity of metal ions

By

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Abstract

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Melanin has capacity to accumulate metal ions in vivo and in vitro. In the present study the relative melanin-affinity of various metal ions has been determined in vitro by use of the bisquaternary ammonium compound paraquat as reference ion. Paraquat has previously been shown to bind to melanin with ionic binding as the dominant mechanism of interaction. The relative melanin-affinity of the metal ions was determined by studying the ability of the metal ions to compete with paraquat for combining ith the melanin. Pigment from beef eyes and synthetic melanin prepared from L-DOPA with tyrosinase are used. The affinity for melanin of the metal ions increased with increasing valencies. The order of affinity of the alkali metals was Cs^+ Rb^+ K $> Na^+ > Li$ and of the alkaline earth metals Ba^{++} $Sr^{++} > Ca^{++} > Mg^{++}$. Tl^+ had slightly higher affinity than the alkali metals. Pb^{++} had the strongest affinity of the divalent cations and Cu^{++} was next in order. Ni^{++} Co^{++} and Mn^{++} showed affinities in the mentioned order. La^{+++} and Gd^{+++} hich are chemically similar although the former is diamagnetic and the latter paramagnetic, showed similar affinity for the melanin, suggesting that it is the chemical and not the magnetic properties which determine the affinity. The studied metal ions showed the same order of affinity for the synthetic melanin as for the eye-melanin, suggesting that the protein moiety of the pigment granules plays minor role in the binding of the metal ions. Melanin has been shown to contain numerous free carboxyl groups. The results are consistent ith the hypothesis that the metal-ion binding of the melanin can be ascribed to cation-exchange activity ith the free carboxyl groups in the melanin polymer as the main binding-sites.

Key words: melanin, cation exchanger metal ions, paraquat

Melanin has a capacity to bind metal ions *in vivo* and *in vitro* (Nicolaus 1962, Cotzias and Papavasiliou 1964 Kikkawa *et al.* 1955 Seiji *et al.* 1963, Stein 1955, Garner 1959 Taylor *et al* 1964 Potts and Au 1971 Bruenger *et al* 1967 Potts and Au 1976). This binding has been ascribed to a cation-exchange activity of the melanin (Bruenger *et al.* 1967 Potts and Au 1976), which in turn may be related t the presence of free carboxyl groups in the melanin polymer (Nicolaus 1968). However data on the relative affinity of various metal ions for melanin are still scarce and it has not been established whether there is a parallelism between the metal-binding capacity of melanin and of other carboxyl ion exchange resins. We have recently shown that the bisquaternary ammonium compound paraquat is bound on melanin in vivo and in vitro with ionic binding as the dominant mechanism of the interaction (Larsson *et al.* 1977). Spectrophotometric determinations of the melanin-binding of paraquat

present in incubation media together with metal ions also gave information on the relative affinity of the metal ions for melanin It became apparent that paraquat may be used as a reference ion to determine the relative affinity of metal ions for melanin. Methods based on the same principle, *i.e.* competition between organic and inorganic cations for the binding to a polyanion, have previously been used to study the relative affinity of cations to polyanions (Scott 1973 Simkiss and Tyler 1958) In the present investigation, the relative affinity of metal ions for pigment from beef eyes and for synthetic melanin prepared from L DOPA has been determined by use of paraquat as a reference ion.

Material and methods

Chemicals. Crystalline paraquat dichloride (N N -dimethyl-4 4 -dipyridylium dichloride) was a gift from the Imperial Chemical Industries Ltd., Alderley Park, Macclesfield, Cheshire England Other chemicals used in the study were of analytical grade and were purchased from regular commercial sources.

Pigment preparation Pigment from beef eyes was prepared as described by Potts (1964). The final pigment granule suspension was adjusted to contain 10 mg by dry weight of pigment granules per ml suspension. It was stored at +2°C until used

Synthetic melanin was prepared by dissolving 1 0 g L DOPA and 20 mg tyrosinase (polyphenol oxidase from mushrooms, 700 units/mg, Sigma Chemical Co., St. Louis, Missouri, USA) i 500 ml 1/15 M Sorensen buffer pH 6.8 After shaking in a water bath t 37°C for 17 h, during which period the solution was bubbled with air the resulting black precipitate was collected by centrifugation and washed 4 times in distilled wate The yield wa 29 The final melanin suspension was adjusted to contai 5 mg by dry weight of melanin per ml suspension. It was stored at +2°C until used.

Experiments The bility of metal ions t compete with paraquat for combination with melanin as studied by adding various amounts of the chloride salts of the metal ions to the incubation solutions containing melani and paraquat. The amount of paraquat which had been bound on the melanin after 45 minutes incubation was then determined. The incubation procedure described by Potts (1964) was used with modifications (Larsson *et al.* 1977). The melanin suspension media contained 10 mg pigment granules or 5 mg synthetic melanin, 25 μmol paraquat (= 3 57 10^{-4} M paraquat) and the desired amount of metal chloride salt in 7 ml of distilled water After the incubations, the pigment was sedime ted by centrifugation at 35 000 × *g* for 10 min in an MSE 25 H gh Speed centrifuge and the paraquat remaining in the supernatant was measured spectrophotometrically in a H tachi Perkin-Elmer 124 Spectrophotometer t 257 nm. All determinations were made i duplicate. The difference between the two determinations was found to be less than ±0.2% Since variations in pH may nfluence the binding of paraquat on melanin (Larsson *et al.* 1977), the salts were also added to distilled wate the same concentrations as used n the incubation experiments, and the pH values of these solutions w re recorded by means of a PHM 62 Standard pH-meter with a glass electrode (Radiometer Copenhagen, Denmark).

Results

Experiments with eye-melanin. In initial experiments, the effect of various concentrations of Pb^{++} Ca and Mg on the binding of paraquat on melanin was determined It was found that the binding of paraquat on melanin was strongly influenced by the ionic strength of the incubation media (Fig. 1) Pb^{++} was more effective than Ca^{+} which in turn was more effective than Mg^{++} at all the concentrations studied On basis of these experiments it was decided to study the inhibiting effect on the melanin binding of paraquat by the various metal ions in concentrations of 3.57 10^{-4} M and 10^{-4} M It was found that in general the extent of inhibition of the paraquat binding increased with increasing valencies of the metal ions (Table I) This was seen both at the high and low concentrations of the metal ions. The

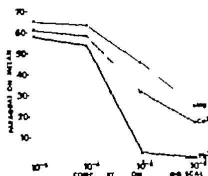


Fig. 1. The ability of Mg^{++}, Ca^{++} and Pb^{++} to inhibit the binding of paraquat on eye-melanin. 2.5 μmol of paraquat (= 3.57 10^{-4} M) was incubated in 7 ml pigment granule (10 mg) suspensions containing various amounts of the chloride salts of Mg^{++}, Ca^{++} and Pb^{++}. After 45 min incubation, the binding of the paraquat on the pigment granules was determined.

order of inhibition among the various metal ions was also the same at both concentrations. Among the monovalent metal ions, Tl was slightly more effective than the alkali metals. The order of effectiveness of the alkali metals was Cs >Rb+>K >Na Li+. Of the divalent metal ions, Pb^{++} and Cu^{++} in the mentioned order were more effective in inhibiting the paraquat binding than the other metal ions in this group. The order of effectiveness of

Table 1. The ability of metal ions to inhibit the binding of paraquat on eye-melanin.

Metal ion	% paraquat on melanin in 10^{-3} M metal ion solution	pH of 10^{-3} M metal ion solution	% paraquat on melanin in 3.57 10^{-4} M metal ion solution	pH of 3.57 10^{-4} M metal ion solution
—*	68.5	6.0**	68.5	6.0**
Li^+	63.3	5.5	67.2	5.6
Na^+	59.7	5.7	66.8	5.7
K^+	58.3	5.5	66.5	5.6
Rb^+	57.2	6.3	65.9	5.9
Cs^+	52.6	5.7	65.8	6.2
Tl^+	—***		59.8	5.9
Mg^{++}	24.8	5.4	54.7	5.4
Ca^{++}	17.0	6.0	45.6	5.7
Sr^{++}	14.2	5.4	45.3	5.3
Ba^{++}	12.5	5.4	41.3	5.4
Mn^{++}	17.7	5.4	43.6	5.5
Co^{++}	14.1	5.6	39.6	5.4
Ni^{++}	11.2	5.4	37.0	5.5
Cu^{++}	4.2	5.0	32.0	5.4
Pb^{++}	0.6	5.2	26.3	5.3
La^{+++}	3.9	5.4	20.3	5.4
Gd^{+++}	3.0	6.5	18.7	5.5

* control, no metal ion added

** pH of distilled water

*** not soluble in 10^{-3} M concentration

2.5 μmol of paraquat (= 3.57 10^{-4} M) was incubated in 7 ml pigment granule (10 mg) suspensions containing 10^{-3} M or 3.57 10^{-4} M metal-chloride salts. After 45 minutes incubation, the binding of the paraquat on the pigment granules was determined.

TABLE II The ability of metal ions to inhibit the binding of paraquat on synthetic melanin.

Metal ion	% paraquat on melanin in 3.57 10⁻⁴ M metal ion solution
—	98.7
Na	98.0
Ca	85.3
Ni^{2+}	72.9
Pb^{2+}	44.1
La^{3+}	23.3

control no metal ion added

2.5 μmol of paraquat (= 3.57 10^{-4} M) was incubated in 7 ml of suspensions containing 5 mg synthetic melanin and 3.57 10^{-4} M of metal-chloride salts. After 45 minutes incubation, the binding of the paraquat on the melanin was determined

the alkaline earth metals was $Ba^{++} > Sr$ $> Ca^{++} > Mg^{+}$ The order of the other divalent metal ions was Ni $>$ Co $>$ Mn La and Gd showed a similar ability to inhibit the melanin binding of the paraquat

The pH values of the solutions of the studied metal ions showed only small variations (Table I). It was obvious that these variations had no relation to the inhibiting capacity of the metal ions. Originally Cr^{3} Al and Sn + were included in the study It was found, however that these cations by hydrolysis lowered the pH They will therefore be present in the water as complex ions with water molecules and OH The ability to inhibit the paraquat binding will then be caused by the complex ions and the H ions released by the hydrolysis and not by the pure metal ions. These metal ions were therefore excluded from the study

Experiments with synthetic melanin Paraquat was more effectively bound on the synthetic melanin than on the eye melanin (Table II) As with the eye melanin, the metal ion induced inhibition of the paraquat binding increased with increasing valencies of the metal ions. The order of effectiveness of the studied metal ions was the same as that observed with the eye-melanin

Discussion

The results of the present study have shown that the attraction to the melanin of the metal ions increases with increasing valencies. This is a general characteristic of cation exchange reactions and is due to the electrostatic fields of the cations (Scott 1968) The order of affinity for the alkali metals was found to be Cs $>$ Rb $>$ K $>$ Na $>$ Li and for the alkaline earth metals Ba $> Sr^{++} >$ Ca + $>$ Mg The adsorbability of these ions is related to their hydrated ionic radii and follows the Hofmeister or lyotrophic series, which is a general order obeyed for many ion exchange systems (Kunin 1958). Bungenberg de Jong (1949) studied the affinity of metal ions for carboxyl colloids (Na-arabinate and Na-pectinate), using the so called reversal-of-charge technique and found the order of affinity of the alkali metals to be K $>$ Na $>$ Li^{+} and for the alkaline earth metals Ba $> Sr^{++}$ Ca Mg Haug (1961) who worked with alginates (which carry carboxyl groups) using H as reference ion, found the same order of affinity for the alkaline earth metals. Thus, these findings correspond

to observations made in the present study. In their studies, Bungenberg de Jong (1949) and Hang (1961) found that of the divalent cations Pb^{2+} had the strongest affinity and that Cu^{2+} was next in order. These findings also correspond to the results of the present study. Bruenger *et al.* (1967) have reported a strong affinity for melanin of ^{210}Pb. A strong affinity of Co for melanin was observed by Potts and Au (1976). Synthetic carboxyl resins have been reported to have a high affinity for Cu^{2+} (Kunin 1958). It has been shown that melanin contains a stable free radical (Commoner *et al.* 1954). Blois *et al.* (1964) showed that Cu^{2+}, which is paramagnetic, eliminated the free radical signal. In the present study the melanin affinity of La^{3+} and Gd^{3+} was studied. La^{3+} is diamagnetic and Gd is paramagnetic. Sarna *et al.* (1976) showed that the free radical signal of melanin is blocked by Gd^{3+} but not by La^{3+}. Since La^{3+} and Gd^{3+} have similar chemical properties and Gd^{3+} and Cu^{2+} have similar magnetic properties, it was concluded that magnetic and not chemical interaction explains the loss of free radical signal (Sarna *et al.* 1976). Studies by Baldry and Swan (1977) indicated that the free radicals of melanin were inaccessible to chemical attacks by reagents such as metal ions. In the present study La^{3+} and Gd^{3+} showed similar affinity for melanin. This indicates that it is the chemical and not the magnetic properties of the metal ions which determine the extent of the binding to the melanin. This result supports the conclusions of Sarna *et al.* (1976) and Baldry and Swan (1977).

The experiments with synthetic melanin showed that the studied metal ions had the same order of affinity as for eye-melanin. This suggests that the protein moiety of the melanin plays a minor role in the binding of the metal ions.

The results of the present study support the hypothesis that the metal ion binding of melanin is determined mainly by the free carboxyl groups present in the melanin polymer and melanin will in this respect behave like a weak acid cation-exchanger. However, a high affinity of a metal ion for melanin *in vitro* does not necessarily mean that a high uptake occurs also *in vivo*. Melanin is an intracellular constituent, and transport barriers at the cell membranes may prevent metal ions from reaching the melanin *in vivo*. Inversely, an effective transport mechanism may promote the uptake in the melanin containing tissues.

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Sympathetic innervation of the urinary bladder and urethral muscle in the pig

By

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Abstract

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The sympathetic innervation of the lower urinary tract was studied in the female and male pig using a specific histochemical technique for visualization of noradrenaline containing nerves. In smooth muscle like the detrusor few evenly distributed adrenergic nerve terminals were found. A greater number of terminals were observed in the trigone, bladder neck and urethra. There was no sex difference. In smooth muscle specimens from pig detrusor and trigone sympathetic nerve terminals were more abundant than in corresponding tissue from humans.

Key words: Pig, urinary bladder, sympathetic innervation, fluorescence histochemistry

The autonomic control of the function of the lower urinary tract is very complex. There are at least twelve pelvic reflexes which are important for normal continence and micturition mechanisms (Mahony *et al.* 1977). Sympathetic as well as parasympathetic nerves are involved in these reflexes. Adrenergic and cholinergic nerve terminals have been demonstrated in the smooth muscle of the bladder and urethra in different species, including man, by means of specific histochemical techniques (El-Badawi and Schenk 1966, Norlén *et al.* 1976, Ek *et al.* 1977 a, Sundin *et al.* 1977). Studies of adrenoceptors carried out *in vivo* and *in vitro* in several animal species and in man have revealed the distribution of and β-adrenoceptors in these organs (Edvardsen and Setekleiv 1968, Awad *et al.* 1974, Ek *et al.* 1977 b, Hardemark *et al.* 1977). Significant species variations have been shown indicating differences in the functional importance of the sympathetic nervous system in the lower urinary tract from animal to animal and from animal to man (El-Badawi and Schenk 1966, Edvardsen and Setekleiv 1968, Nergårdh *et al.* 1977). Recently the pig has, with advantage, been taken into use for experimental purposes in studies of the kidney, ureter and bladder (Gyrd-Hansen *et al.* 1969, Djurhuus *et al.* 1976, Larsen and Mortensen 1978). The ultrastructure of the detrusor muscle of the pig has been described by Larsen (1977), and the

TABLE I Evaluation of the sympathetic innervation of the smooth muscle in the pig lower urinary tract (n = 10) based on the distribution of adrenergic nerve terminals according to the following score system

0 No adrenergic nerve terminals observed in the muscle.
1: Less than one adrenergic nerve terminal present per smooth muscle bundle.
2 On the average one adrenergic nerve terminal in each smooth muscle bundle.
3 On the average 2 5 adrenergic nerve terminals in each smooth muscle bundle
4 On the average one adrenergic nerve terminal at each smooth muscle cell (*i.e.* 5–10 nerve terminals per muscle bundle) corresponding to the picture seen i control sections obtained from the ductus deferens of the rat.

Pig no.	1	2	3	4	5	6	7	8	9	10	$\bar{x}$
Sex	♀	♀	♀	♂	♂	♀	♂	♀	♀	♀	
Detrusor anterior	1	1	1	1	1	1	1	2	2	2	1.3
Detrusor right side	0	1	1	1	1	1	1	2	1	1	1.0
Detrusor left side	1	1	1	1	1	2	2	1	2	1	1.3
Detrusor posterior	1		1	0	1	1	2	1	1	1	1.0
Trigone area	3	3		2	2	3	3	2	3	3	2.6
Bladder neck	1	3	4	1	4	)	3	3	3	3	2.8
Urethra		)	3	)	3	3	3	2	3	3	2.8

) Specimen with too small amount of smooth muscle for evaluation.

presence and type of β-adrenoceptors in the detrusor muscle of pig and man have been investigated (Larsen 1978) The present study was carried out in order to evaluate quantitatively the morphology of the sympathetic innervation of the lower urinary tract of the pig and to compare the results with previously reported findings in man (Nordling and Christensen 1978).

Methods

Specimens from seven female and three male (boars) pigs weighing about 90 kg were obtained at a slaughter house The bladder and urethra were removed n toto, in ine cases 25 ml and in one case (pig no. 1) immediately after death. Specimens f smooth muscle were removed from the anterior posterior and lateral walls of the detrusor from the trigone bladder neck and urethra just above the pelvic floor The muscle pieces were frozen liquid propane cooled by liquid nitrogen according to the method described by Falck *et al.* (1962) The specimens were freeze-dried for 5–7 days, then exposed to formaldehyde vapour at 80°C for 1 h, imbedded in paraffin and cut in 10 μm thick sections. During this treatment catecholamines are converted i to strongly green fluorescent products (Falck *et al.* 1962). Sections were investigated by fluorescence microscopy before and after addition f freshly prepared 0.03 sodium borohydride ($NaBH_4$) in 95% isopropyl alcohol. Addition of NaBH causes the specific catecholamine fluorescence to disappear within a few seconds—a process which can be observed in the microscope By this method the specific catecholamine fluorescence can be distinguished from the persistent non-specific fluorescence (g auto-fluorescence) of elastic fibres etc. (Corrod t al. 1964 Nordling nd Christensen 1978).

A Leit orthoplan fluorescence microscope supplied with n ultra high pressure mercury lamp (Osram HBO 200) and equipped with a Leitz excitation filte BO 3 + S 405 nd an emission filter K 490 was used. Photomicrographs were taken on Kodak Pl Estar AH 35 mm roll films w th an exposure time from 20 to 30 s. The processi g of the films was mod fied to give film sensitivity equivalent to 800 ASA

A score system was used to quantitate the distribution of adrenergic innervation (see legend t Table I).

Results

Table I shows the results obtained according to the score system Adrenergic nerve terminals were observed in the smooth muscle of all parts of the lower urinary tract examined. There

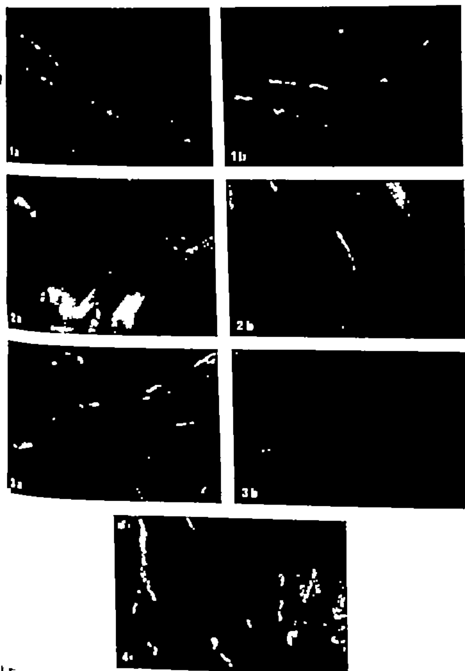


Fig. 1. Smooth muscle from the urinary bladder neck of female pig (a) and male pig (b). Some adrenergic nerve terminals are present. Fluorescence macrophotography × 300.

Fig. 2. Smooth muscle from the anterior part of the detrusor of pig no. 1 ( ) and pig no. 2 (b). An adrenergic nerve terminal is present in each picture. Fluorescence macrophotography × 300.

Fig. 3. Smooth muscle from the trigone area before (a) and after (b) the addition of sodium borohydride. The disappearance of adrenergic nerve terminals is illustrated. Fluorescence macrophotography × 300.

Fig. 4. Smooth muscle from the proximal part of the urethra. Some adrenergic nerve terminals are present. Fluorescence macrophotography × 300.

TABLE I Evaluation of the sympathetic innervation of the smooth muscle in the pig lower urinary tract (n = 10) based on the distribution of adrenergic nerve terminals according to the following score system

0: No adrenergic nerve terminals observed in the muscle.
1: Less than one adrenergic nerve terminal present per smooth muscle bundle.
2 On the average one adrenergic nerve terminal in each smooth muscle bundle.
3 On the average 2 5 adrenergic nerve terminals in each smooth muscle bundle
4 On the average one adrenergic nerve terminal at each smooth muscle cell (i.e 5–10 nerve terminals per muscle bundle) corresponding to the picture seen in control sections obtained from the ductus deferens of the rat.

Pig no.	1	2	3	4	5	6	7	8	9	10	$\bar{x}$
Sex	♀	♀	♀	♂	♂	♀	♂	♀	♀	♀	
Detrusor anterior	1	1	1	1	1	1	1	2	2	2	1.3
Detrusor right side	0	1	1	1	1	1	1	2	1	1	1.0
Detrusor left side	1	1	1	1	1	2	2	1	2	1	1.3
Detrusor posterior	1		1	0	1	1	2	1	1	1	1 0
Trigone area	3	3		2	2	3	3	2	3	3	2 6
Bladder neck	1	3	4	1	4	)	3	3	3	3	2.8
Urethra	2	)	3	)	3	3	3	2	3	3	2.8

) Specimen with too small amount f smooth muscle for evaluation

presence and type of β-adrenoceptors in the detrusor muscle of pig and man have been investigated (Larsen 1978) The present study was carried out in order to evaluate quantitatively the morphology of the sympathetic innervation of the lower urinary tract of the pig and to compare the results with previously reported findings in man (Nordling and Christensen 1978)

Methods

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A Leitz orthoplan fluorescence microscope supplied with an ultra high pressure mercury lamp (Osram HBO 200) nd equipped w th Leitz excitation f lter BO 3 + S 405 and an emission filter K 490 as used. Photomicrographs were taken on Kodak Pan Estar AH 35 mm roll films with an exposure time from 20 t 30 s. The processing of the films was modified t give film sensitivity equivalent t 800 ASA.

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Results

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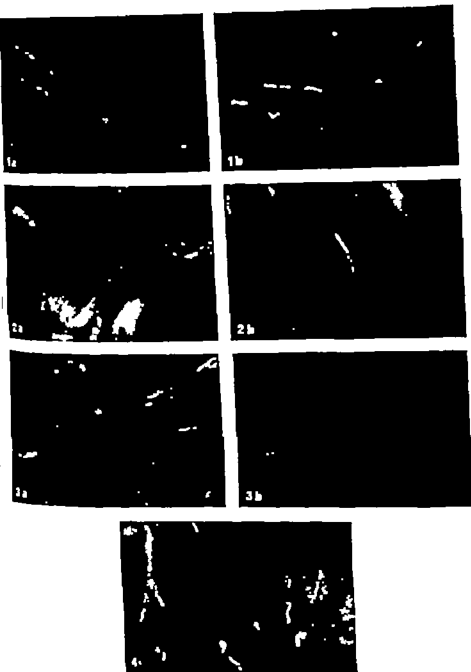


Fig. 1. Smooth muscle from the urinary bladder neck of female pig ( ) and male pig (b). Some adrenergic nerve terminals are present. Fluorescence macrophotography 300.

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Fig. 3. Smooth muscle from the trigone area before (a) and after (b) the addition of sodium borohydride. The disappearance of adrenergic nerve terminals is illustrated. Fluorescence macrophotography 300.

Fig. 4. Smooth muscle from the proximal part of the urethra. Some adrenergic nerve terminals are present. Fluorescence macrophotography 300.

was no difference between female and male pigs in any of the regions investigated (Table I and Fig. 1 a and 1 b).

No visible difference in number of nerves or intensity of fluorescence was seen between specimens obtained immediately after the death of the animal and specimens obtained 25 min after killing (Fig. 2 a and 2 b).

In spite of some variation the sympathetic nerves were clearly more abundant in the smooth muscle of the trigone (Fig. 3 a), bladder neck (Fig. 1 a and 1 b) and urethra (Fig. 4) than in the detrusor muscle (Fig. 2 a and 2 b). The number of adrenergic nerve terminals was almost equal in the trigone, bladder neck and urethra. The distribution of the sympathetic innervation of the detrusor muscle was without regional variations.

Generally the adrenergic nerve terminals in the smooth muscle examined were running parallel to the muscle cells. Transverse or oblique orientation was seldomly observed. Sympathetic terminals were abundant in the smooth muscle of blood vessels, but this innervation was carefully excluded in the present evaluation. No ganglionic cells were seen in any of the preparations.

Discussion

Differentiation between catecholamine fluorescence and autofluorescence based on the morphology and colour of the fluorescent product was found to be uncertain. However by using NaBH (Fig. 3 a and 3 b) an evaluation of the distribution of the adrenergic innervation based on the observation of the specific catecholamine fluorescence was considered to be reliable.

In a previous work it has been shown that the smooth muscle of the human detrusor and trigone has a very scarce sympathetic innervation (score 0 with the present score scale) (Nordling and Christensen 1978). Others have reported the same finding in smooth muscle of human detrusor, trigone and urethra (Ek *et al.* 1977 a, Sundin *et al.* 1977, Gosling and Dixon 1977) although some reported more abundant adrenergic nerves in the trigone (Ek *et al.* 1977 a, Sundin *et al.* 1977) and preprostatic male urethra (Gosling and Dixon 1977).

Species differences in the sympathetic innervation of the lower urinary tract have been demonstrated by several workers. El-Badawi and Schenk (1966) showed a pronounced difference in the distribution pattern of the adrenergic nerve terminals in the smooth muscle of the bladder from cat and dog on the one hand and rat and guinea-pig on the other hand. The number and general distribution of adrenergic nerves in the human bladder reported by Ek *et al.* (1977 a) and by Sundin *et al.* (1977) has by the former workers been found in accordance with the findings in animal species such as cat, dog, rabbit, rat and guinea-pig (Hamberger and Norberg 1965, El-Badawi and Schenk 1966, Owman and Sjöberg 1972, Wakade and Kirpekar 1972, Raezer *et al.* 1973, Sundin and Dahlström 1973, Dixon and Gosling 1974, Alm and Elmér 1975, Raezer *et al.* 1976). The scarce sympathetic innervation found in the human urethra (Ek *et al.* 1977 a) differed from the rich one in *e.g.* cat, dog, guinea pig and rat (Owman *et al.* 1971, Owman and Sjöberg 1972, Sundin and Dahlström 1973, Benson *et al.* 1976). The very scarce sympathetic innervation of the smooth muscle of the human bladder neck and female proximal urethra found by Gosling *et al.*

(1977) differed from the rich one found in the cat, dog, rabbit and rat (Hamberger and Norberg 1965, El-Badawi and Schenk 1974, Gosling and Dixon 1975). It should however be mentioned that in most of the cited studies no scoring system for quantitation of the innervation has been used, which makes comparisons difficult.

The finding of a scarce but constant amount of adrenergic nerves in the detrusor muscle and a rich supply in the trigonal muscle of the pig differs from previous findings in human material using exactly the same technique (Nordling and Christensen 1978). In this study no score system was applied mainly because the adrenergic nerve supply of the smooth muscle was so scarce that all preparations would have obtained score 0 with the score scale used in the present study.

It can therefore be concluded that the adrenergic nerve supply of detrusor and trigone smooth muscle is more abundant in the pig than in the human. Experimental results from the pig concerning physiology of the lower urinary tract, especially in respect to sympathetic innervation, can therefore only with great caution be extended to human physiology.

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Effect of synthetic substance P on internal carotid artery blood flow in man

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Abstract

Sandegård, H., L. Thulin, G. Tydén, C. Johansson, O. Muhrbeck and C. Björklund. *Effect of synthetic substance P on internal carotid artery blood flow in man.* Acta physiol. scand. 1978. *104* 491–495

Internal carotid artery blood flow was studied by peroperative electromagnetic blood flow measurement in 7 patients following 9 intravenous infusions of synthetic Substance P 2 ng kg^{-1} min^{-1}. The blood flow response varied interindividually. No significant blood flow changes were observed for the whole group. The arterial mean blood pressure decreased significantly.

The circulatory effect of substance P (SP) has been extensively studied both in animals and man since the initial report on SP was published by Euler and Gaddum (1931). SP has been found to influence the circulation with a transient fall in arterial blood pressure, both in animals and in man (Euler and Gaddum 1931, Pernow 1953 (a), Löfström *et al.* 1965, Brownell 1968, Hallberg and Pernow 1975, Burcher *et al.* 1977, Eklund *et al.* 1977, Melchiorri *et al.* 1977). The fall in pressure is the result of a peripheral vasodilatation with increased blood flow in skin, muscle, adipose tissue (Pernow and Rosell 1975) and in the splanchnic vessels (Hallberg and Pernow 1975). Substance P appears to act directly on the vascular smooth muscle (Löfström *et al.* 1965, Eklund *et al.* 1977).

The occurrence of SP in the brain tissue (Pernow 1953 (b), Amin *et al.* 1954, Arnautović *et al.* 1963, Laszlo 1963, Baldauf *et al.* 1968, Otsuka *et al.* 1975, Mroz *et al.* 1977, Hökfelt *et al.* 1977), and its potent vasodilating effect has focused our interest on the effect of i.v. administered synthetic SP on the internal carotid artery blood flow in man. A small pilot study indicated that internal carotid artery blood flow increased in normotonic patients following SP (Thulin *et al.* 1977). The present work reports an extended investiga-

Material

The investigation was performed in 7 patients (5 males and 2 females) with a mean age of 67 years (range 59–72 years), who were accepted for internal carotid artery (ICA) surgery. Indication for operation as based on clinical symptoms, mostly transient ischemic attacks, and on angiographical findings of obstructive disease of the carotid bifurcation. All patients suffered from arteriosclerosis. In 3 patients the ICA operated upon was stenosed by more than 50 per cent of the diameter of the lumen. Three patients suffered from hypertensive disease. No one had previously suffered from cardiac infarction or atrial fibrillation.

Methods

Internal arotid artery mean blood flow ml/min, was measured peroperatively with the aid of a squarewave electromagnetic flowmeter. An extravascular shunt (Ekeström 1968) was used during the reconstruction. The blood measurements were performed after the arterial reconstruction during neurolept anesthesia with N_2O O_2, fentanyl nd droperidol.

The flowprobe was placed on the common carotid rtery and the external carotid nd the superior thyroid arteries were occluded during measurement. Zero flow line was obtained by occlusion of the vessel.

Brachial artery mean blood pressure mmHg, was measured by a mechano-electric pressure transducer. Both ICA flow and arterial blood pressure were continuously registered on a direct nting recorder.

Arterial carbon dio ide t nsion, ($PaCO_2$), kPa, was measured with the aid of a Severinghaus electrode. It was controlled every minute d ring measurements and kept constant by artificial entilation.

Synthetic Substance P (Fisher *et al* 1974) dosage ng kg⁻ min was given i.v. duri g 1 min. ICA mean blood flow and arterial mean blood pressure were continuously measured during 5 min.

Statistical methods

Standard methods for st tistical calculations of the data were employed (Snedecor nd Cochran 1967). The following probability (p) levels of significance were used $p<0.001$ $p<0.01$ $p<0.05$

Results

ICA blood flow arterial mean pressure and PaCO were studied in 7 patients after 9 i.v infusions of SP. The results are presented in Fig. 1.

ICA mean blood flow was 188 ± S D 59 ml/min before administration of SP. The ICA blood flow increased after 4 infusions of 2 ng kg min of SP and decreased or remained unchanged after 5 infusions. The mean ICA blood flow for the whole group was not significantly changed during the 5 min studied. There was no difference between hypertensive or normotensive patients.

Arterial mean blood pressure was 129 ± S D 27 mmHg before SP administration and decreased significantly within 1 min to 110 ± S D 40 mmHg ($p<0.01$). The arterial me. pressure returned to control level within 2 min.

Arterial carbon dioxide tension was 4 0 ± S D 0 6 kPa. It was not significantly change during the procedure.

Discussion

As far as known, there are no reports in the literature concerning the influence of SP o cerebral blood flow (CBF) or ICA blood flow in man except for the previously mentione pilot study (Thulin *et al* 1977).

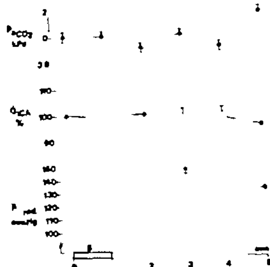


Fig. 1. Arterial CO_2 tension ($PaCO_2$), kPa, ICA mean blood flow ($\dot{Q}_{ICA}$) as per cent of control flow and radial artery mean blood pressure ($\bar{P}A$ rad.), mmHg, before and after intravenous administration of 2 ng kg^{-1} min^{-1} of SP during 1 min. Vertical range bars indicate standard error of mean. Asterisk indicate probability levels of significance (p)

Electromagnetic flowmetry after arterial reconstruction of the carotid artery has been used to describe the interaction between central hemodynamics and cerebral circulation as reflected by ICA blood flow when arterial carbon dioxide tension, posture and physical activity are varied (Sannegård 1974). We have used this method peroperatively since the ICA flow changes most probably reflect changes in the cerebral blood flow of the tissue supplied by the artery (Sannegård and Carlens 1974). Furthermore, electromagnetic flowmetry allows registration of very rapid and brief flow changes.

Hallberg and Pernow (1975) studied the effect of SP on common carotid artery flow in the dog. They found about 50 per cent flow increase after continuous infusion of 2.4–3.6 μg kg^{-1} min^{-1} of SP. This includes flow changes both in the internal and external carotid artery. In the dog only a minor part of the common carotid artery blood flow supplies the brain. Thus, the flow increase noticed by these authors may be the result of dilatation of the vessels to muscles and skin of the skull and no conclusions concerning the flow to the brain can be drawn.

The responses in blood pressure and ICA blood flow following SP varied considerably between the different patients. In 3 patients the arterial mean blood pressure was unchanged. In two of them the ICA blood flow remained unchanged and in one the blood flow decreased slightly. In two others ICA flow decreased by 15–20 per cent, presumably secondary to a pressure drop down to 55–70 mmHg, which values appear to be below the pressure level for cerebral circulatory autoregulation (Lassen 1959). In two patients ICA blood flow increased 13 per cent in spite of a decrease in arterial mean pressure to 90 mmHg from 110 and 125 mmHg respectively. The ICA blood flow increase in these patients indicates true vasodilatation in the cerebral vascular bed. But, on the other hand, the ICA flow decreased more than 10 per cent in two patients without significant pressure changes, suggesting a vasoconstriction.

The well documented vasodilatating effect of SP on the peripheral vascular bed was also found in this study by a significant decrease in mean arterial blood pressure for the whole

group following infusion of SP If SP has a direct effect on the vascular smooth muscle (Löfström *et al* 1965 Eklund *et al* 1977) a dilatation of the cerebral vessels with CBF increase would be expected Minor increase in total CBF does not necessarily result in significant increase in ICA blood flow but a flow reduction as found in some patients, is definitely not expected.

Judging from the present study SP has a vasodilatating effect in the peripheral vascular bed, but no similar effect on the cerebral vascular bed. The previous observations of a possible role for SP as a cerebral vasodilator must therefore be considered less significant.

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Acta physiol. scand 1978. 104 496–498
From the Department of Physiology University of Göteborg, Sweden

Acute effects of pressure on resistance vessel geometry

By

Björn Folkow

The structurally set dimensions of inner radius (r_i) and wall thickness (w) are obviously of major importance for resistance vessel function, and even more so the ratio between them, w/r_i, particularly concerning the altered hemodynamics in hypertension (e.g. Folkow 1956, 1978) It is known since over 100 years that hypertension is associated with hypertrophic thickening of the bulky arteriolar media, a microvascular analogue to left ventricular hypertrophy However the hemodynamic importance of this structural adaptation was for long overlooked, partly because it could first recently be precisely measured. The reason is that even modest shifts in smooth muscle activity and distending pressure, most difficult to avoid for microvessels, affect both w and r_i though in opposite directions, implying profound w/r_i changes and hence great errors.

It was first when techniques were introduced for standardization of the w/r_i balance that precise morphometric arteriolar comparisons became possible, thanks to Short (1966) Furuyama (1962) and Suwa and Takahashi (1971) When combined, their measurements in human primary hypertension show a structural r_i reduction associated with a media adaptation, so as to increase the w/r_i ratio largely in proportion to the pressure elevation These changes were more marked in splanchnic and renal vessels than in muscle ones Further close to the capillary level the increased w/r tapers off suggesting that the smallest precapillary vessels are partly protected from the pressure elevation thanks to the increased 'upstream resistance Recently Mulvany Aalkjaer and Hansen (1978) arrived at similar results on isolated mesenteric arterioles from spontaneously hypertensive rats (SHR) and normotensive controls (NCR) Measured at equal distending pressures and complete relaxation, the media thickness was increased about 50 per cent and r_i reduced 15 per cent in SHR, if anything elevating the structurally set w/r_i *more* than pressure was raised in this vascular bed like in man — These morphometric findings fully confirm hemodynamic estimations of the altered resistance vessel design in hypertensive man and rats showing, among other tings, a structurally determined average r_i reduction, despite exposure to a raised distending pressure (Folkow 1956 1978)

However recently Bohlen and Lobach (1978) performed *in vivo* morphometric estimations of r_i and w in a skeletal muscle (cremaster) vascular bed of SHR and normotensive controls (WKY). Though results varied between consecutive vascular sections, there was on an average no clear signs of any structural w/r_i increase even in established SHR hypertension. It is not unlikely however that the distal microvessels studied ($r_i \sim 50$ µm or below)

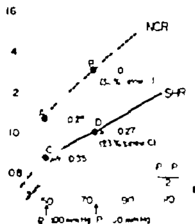


Fig. 1. Relationship between distending pressure and calculated average r_i shifts for SHR and NCR resistance vessels, at identical levels of modest vascular smooth muscle activity but exposed to sudden pressure changes (redrawn from experimental data by Hallbäck, Lundgren and Weiss, Fig. 2). The resulting changes in w/r relationships are inserted along the distensibility curves for . Calculated relative resistances to flow are at A 100, at B 45, at C 225 and at D 135 units.

had in reality been more or less 'protected' from pressure elevation thanks to an increased resistance upstreams (see above), which would correspondingly limit their structural adaptation. Anyhow, the question arises whether these *in vivo* morphometric results diverge from the mentioned morphometric and hemodynamic *in vitro* ones also because of some important technical difference, because of regional differences between systemic circuits, or for other reasons.

This question is of greatest importance for understanding the nature of the raised resistance in hypertension and also for the correct interpretation of hemodynamic and morphometric analyses. There is, however, one important difference between this morphometric *in vivo* comparison and most of the earlier *in vitro* ones. The former was performed with the vessels exposed to the 'ordinary' SHR and WKY pressures of 150 and 100 mmHg, respectively, i.e. at a 50 per cent difference in distending force. As resistance vessels are quite distensible (Hallbäck, Lundgren and Weiss 1974), these unequal pressures may mask a truly existing w/r difference between SHR and NCR by reducing w and increasing r in SHR.

To elucidate this problem calculations were performed based, *first*, on earlier hemodynamic estimations of average r_i values and resistance vessel distensibility in SHR and NCR, *second*, on w/r values closely agreeing with the mentioned *in vitro* morphometric data and with those derived from quantitative hemodynamic analyses in SHR (*cf.* Folkow 1978, Fig. 3). Thus, Fig. 1 reproduces data from Fig. 2 of Hallbäck, Lundgren and Weiss (1974), showing average values deduced from precise pressure-flow measurements ('SHR I' and 'NCR I') in SHR and NCR hindquarter resistance vessels, when kept at equal, steady levels of modest smooth muscle activity but exposed to sudden pressure changes. For the 'model' NCR resistance vessel, with a relative value of 1.07 at 100 mmHg at modest smooth muscle tone and w/r set to 0.25, while the 'model' SHR vessel (r_i 0.87) is assumed to have a 40 per cent higher w/r (0.35) at the same pressure and smooth muscle activity.

From these data, the changes in w/r upon *acute* pressure alterations can be calculated, taking total wall mass as a constant. The results, summarized in Fig. 1, show the great importance of keeping not only smooth muscle activity but also distending pressures rigorously identical, if comparable data for r, w and w/r should be obtained. Thus, while w/r values for SHR are 40 per cent higher than for NCR at 100 mmHg (A vs C),

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Peptide neurons in the vagus, splanchnic and sciatic nerves*

By

JAN M. LUNDBERG, TOMAS HÖKFELT, GÖRAN NILSSON, LARS TERENIUS,
JENS REHFELD, ROBERT ELDE, SAMI SAID

Peripheral nerves in general contain both motor and sensory fibres. The classical transmitter substances in the motor nerves are either acetylcholine (Loewi, 19 1) or noradrenaline (Euler 1956), although a non-cholinergic, non-adrenergic component also has been described (see Burnstock 1977). In addition peptides have been observed *e.g.* in extracts of the vagus nerve, including substance P (SP) (Euler 1963), vasoactive intestinal polypeptide (VIP) (Said and Rosenberg 1976), gastrin (GAS) (Uvnäs-Wallensten *et al.* 1977) and somatostatin (SOM) (Uvnäs-Wallensten *et al.* 1978). In the present communication 3 different types of peripheral nerves were studied by the indirect immunofluorescence technique using antisera to several peptides. With this approach it was attempted to define further the cellular localization of such peptides.

10 male albino rats (b.wt. 150–200 g) and 10 male guinea pigs (b.wt. 300–400 g) were subjected to the following treatments according to the techniques described by Dahlström (1971). The vagus nerve was ligated at the cervical level, the greater splanchnic nerve at the subdiaphragmatic level and the sciatic nerve one cm below the foramen infrapiriforme. In 5 other animals of each kind colchicine (20%) or vinblastine (1%) were applied locally on the nodose ganglion and on the first sacral spinal ganglion. These drugs and the ligation technique were used to increase the levels of the peptides, since in untreated animals the cell bodies and axons often contain too low amounts to be visualized with immunocytochemistry. 24 h after both types of treatment the animals were perfused with ice-cold 4% paraformaldehyde and the nerves and ganglia were processed for the indirect immunofluorescence technique (see Coons 1958). Briefly, cryostat sections were incubated with an antiserum raised to one of the peptides, SP, SOM, VIP, GAS and methionine-enkephalin (met-ENK) followed by a second incubation with fluorescein-isothiocyanate conjugated antibodies and examined in a Zeiss fluorescence microscope. The different antisera pretreated with an excess of the respective peptide served as control sera. For further details on experimental procedures including characteristics of antisera, see Lundberg *et al.* (1979). It should, however, be pointed out that the GAS antiserum also cross-reacts with cholecystokinin (CCK).

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* Part of these results were communicated at the 6th ETP winterschool in Zuoz, Switzerland, January 1978.

and 60 per cent higher at 150 mmHg due to lower vascular distensibility in SHR (B vs D), this w/r_i difference is marginal only (8 per cent) when normotensive vessels at 100 mmHg are compared with hypertensive ones at 150 mmHg (A vs D). The reason is the great reduction of w/r_i when distension increases r_i but decreases w, which also the A/B and C/D relationships illustrate. Resistance is, however, still 35 per cent higher in D vs A.

It might be argued that morphometric comparisons of hypertensive and normotensive vessels should better be performed at their ordinary pressures. However this would easily be misleading, as shown by the over 30 per cent w/r_i reduction and over 20 per cent r_i increase when normotensive vessels are, without other changes, exposed to the 150 mmHg acting on SHR vessels (A vs B). To acutely compensate for this passive 55 per cent reduction of resistance, a considerable smooth muscle activation would be needed in NCR, even to keep resistance the same as at 100 mmHg. The crucial difference to SHR is that the SHR resistance vessels can at 150 mmHg maintain a 35 per cent higher resistance than NCR at 100 mmHg *without* any accentuation of smooth muscle activity (Fig. 1). The more prominent the structural w/r_i elevation, the more marked this ability of hypertensive vessels to maintain a high resistance at low smooth muscle activity where, as mentioned, splanchnic vessels in both man and SHR seem to be even more 'efficient' than muscle ones.

In conclusion the *in vivo* results of Bohlen and Lobach, as compared with those derived from earlier morphometric measurements *in vitro* and from quantitative hemodynamic analyses, may only be apparently in conflict and may, in fact, coincide fairly closely concerning w/r_i values, if only the most important influence of wall distensibility is taken into account. It follows that morphometric measurements must be performed at strictly *equal* distending pressures to allow for direct comparisons of w/r_i values and, further, deal with precisely corresponding precapillary sections, since the structural w/r_i increase tends to taper off close to the capillary level. The results also emphasize the crucial hemodynamic importance of resistance vessel design with respect to its acute and chronic changes upon pressure alterations, and its great relevance for wall tension per unit wall layer.

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met-ENK-like peptide may be contained in axons of motor fibres. The nature of the fibres containing the peptides in the splanchnic nerves is at present under investigation. Similar distribution patterns have also been observed in immunohistochemical studies on these three types of nerves in the cat (Lundberg, Uvnäs-Wallensten, Hökfelt et al. in preparation). Thus, in addition to the previously identified SP and SOM immunoreactive cells in sensory ganglia (Hökfelt et al. 1976) two further subpopulations have been identified containing a GAS-(CCK)like and a VIP-like peptide, respectively. Finally, ENK-like material is present in peripheral nerves with a probable central origin. The localization of the peripheral innervation areas and of putative opioid receptors of such ENK positive fibers projecting e.g. in the sciatic nerve remains to be elucidated.

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Fig. 1 A–C. Immunofluorescence micrographs of sections of the agus nerve (A) and sciatic nerve (B) and the nodose ga glion (C) f the rat 24 h after ligatio (A B) or colchicine application (C) incubated ith ntiserum to SP (A, C) and met ENK (B). Many SP (A) and some met ENK (B) positive fibres are seen above the ligation and some met ENK positive ones below (B). Small and large (arrows I C) SP positive cells are seen in the nodose ganglion. Arrow heads in A and B indicate site of ligation. Bars indicate 50 µm.

SP SOM VIP- GAS- and met ENK immunoreactive material were found in all three nerves studied as shown by their accumulation central (and in the sciatic nerve, partly peripheral) to the ligations, indicating a rapid axonal transport (Fig. 1 A, B). In Table I, subjective estimations have been made to schematically indicate the number of peptide containing nerves above a ligation in the guinea pig. In the rat similar distribution patterns were observed although the number of nerves appeared to be slightly lower. In the nodose ganglion high numbers of small and some large SP immunoreactive cell bodies were observed (Fig. 1 C). Moderate numbers of VIP immunoreactive cells but only few SOM and GAS positive cells could be seen, virtually all being of the small type. No met ENK immunoreactive cells could be demonstrated in the ganglion. In the first sacral spinal ganglion numerous GAS positive neurons, partly of the large type were seen. In addition SP SOM and VIP but no ENK immunoreactive small neurons were observed.

The present results indicate that in the vagus nerve complex as well as in the sciatic nerve SP- SOM (CCK)like peptides, at least partly are present in sensory neurons, whereas a

TABLE I. Subjective estimation f the number of peptide containing axons I the vagus, splanchnic and sciatic nerve of the guinea pig. The estimates are based on analysis f accumulations of immunoreactive material above ligation + + + + + = very high, + + + + = high + + + = medium, + + = low and + = single LI = Like immunoreactivity For bbreviations of peptides see text.

	SP-LI	SOM-LI	VIP LI	GAS-(CCK) LI	met ENK LI
N vagus	+ + + + +	+	+ + +	+	+ +
N splanchnicus major	+ + +	+	+	+	+ + +
N ischiadicus	+ + +	+	+ +	+ + +	+

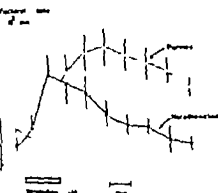


Fig. 1

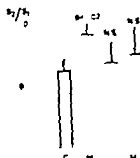


Fig. 2

1 Release of ³H-labelled purines and ³H-labelled noradrenaline from prelabelled rat cortical slices. Data are given as fractional release per min and represent mean ± S.E. of 5 exps.

Fig. 2 Effect of morphine (M), naloxone (N) and the combination on the veratridine induced purine release from cortical slices. The drugs were present at 1 µM concentration between 65 and 106 min after the start of superfusion. Veratridine was present in the superfusion period at 47–50 min (S_1) and at 87–90 min (S_2). Data are given as S_2/S_1 ratios of individual experiments. Mean ± S.E. of 4–6 exps.

...the slices were removed and digested in NCS tissue solubilizer (Amersham, G.B.) and its radioactivity assessed. The fractional release (i.e. the amount released in each collected fraction divided by the amount in the tissue at the time of collection) was calculated.

After some 30 min the rate of efflux of purines reached a steady level of 0.71 ± 0.03 (n=4) % of tissue content per min and after 70 min it was 0.74 ± 0.11 %. The basal rate of efflux was not affected by 1 µM morphine (0.78 ± 0.09), 1 µM naloxone (0.71 ± 0.09) or by morphine-naloxone (0.82 ± 0.1). Between 47 and 50 min after start of superfusion veratridine (... µM) was included in the buffer. As seen in Fig. 1 this caused the release of purines as well as noradrenaline. The release of noradrenaline was abolished by omission of calcium and inclusion of EGTA, while the purine release was more than 50 % reduced. The purine compounds were inosine, adenosine and hypoxanthine predominantly as judged by TLC. Interestingly the release of noradrenaline was much faster in onset than the release of purines (Fig. 1). This could indicate that the mechanism behind the release of the two types of compounds were different, for example that purine release is secondary to the release of a transmitter.

The depolarization-induced release of purines was not completely reproducible. During the first stimulation (47–50 min) 3.4 ± 0.4 % of tissue content was released; during the second (87–90 min) 2.2 ± 0.7 %. Therefore the effect of drugs was tested during the second stimulation period (present from 65 min onwards) and the amount released during this second stimulation (S_2) was divided by that released during the first (S_1) in the same slices. It is seen in Fig. 2 that morphine (1 µM) caused a significant enhancement of the purine release. Naloxone (1 µM) had no effect, nor did the combination of morphine and naloxone.

Thus, the present results demonstrate that morphine increases veratridine induced purine release from rat cortical slices. The effect of morphine was not observed in the presence of

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Morphine increases depolarization induced purine release from rat cortical slices

By

BERTIL B FREDHOLM and LOUISE VERNET

Theophylline antagonizes several different actions of morphine, including analgesia in mi (Ho *et al* 1973) the inhibitory effect of morphine on electrically induced contractions i the guinea pig longitudinal muscle myenteric plexus (Sawynok and Jhamandas 1976), th direct stimulatory effect of morphine (Grubb and Burks 1975), and the inhibitory effect o morphine on acetylcholine from brain slices (Jhamandas *et al* 1978). The mechanism o action of theophylline is not known However theophylline is known to antagonize severa actions of adenosine. For example, the inhibitory effect of adenosine on field stimulate guinea-pig longitudinal muscle is inhibited by theophylline (Sawynok and Jhamandas 1976) Adenosine inhibits acetylcholine and noradrenaline release (Sawynok and Jhamandas 1976 Vizi and Knoll 1976 and this effect is also antagonized by theophylline.

Theophylline thus inhibits effects of both morphine and adenosine, but the two types o agents are not acting on the same receptors, since adenosine effects are not antagonized b naloxone which readily inhibits morphine effects (Sawynok and Jhamandas 1976, Gustafs son, Hedqvist and Fredholm, unpublished data) The data are, however compatible wit the opinion that morphine induces release of adenosine (or a related compound) and that thi accounts at least partly for the effects of morphine. Circumstantial support for such a schem is provided by the report that the inhibitory effect of morphine on contractions of the field stimulated guinea pig ileum is potentiated by dipyridamol (Gintzler and Musacchio 1975), a compound known to potentiate the effects of adenosine by inhibiting its inactivation.

We report the preliminary finding that morphine enhances depolarization induced release of adenosine and other purines from rat cortical slices.

After decapitation the rat brains were rapidly dissected out Thin slices (0 5–1 mm) of the occipital cortex were cut by a razor blade. From the middle of the slice circular section (7 mm diam) was punched out. 4 slices were incubated at 37°C in 5 ml Krebs-Ringer bicarbonat buffer for 40 min w th 10 μCi 2,8-H-adenine ^{14}C-adenine or l-7 ^{3}H-noradrenaline (Radiochemical Centre Amersham). On the verage some 10 nCi was taken up per slice. After washing, the slices ere placed in thermostated plastic chambers and superfused with Krebs-Ringer bicarbonat buffer of the followi g composition (mM) NaCl (118), KCl (4.85), $CaCl_2$ (0.25), + $MgSO_4$ (1 15), KH PO (1 15), $NaHCO_3$ (25) glucose (11 1), pH 7.2 7 4 gassed with 95% O_2 and 5% CO_2.

Samples of the superfusion solution were continuously taken nd the radioactivity counted in scintillation coctail consisting of 4 g/l Omnifluor (Packard) in toluene Triton X 100 (2 1). At the end of the ex

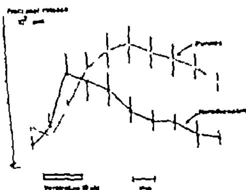


Fig. 1

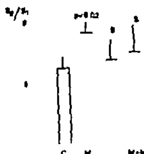


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periment the slices were removed and digested in NCS tissue solubilizer (Amersham, G.B.) and the radioactivity counted. The fractional release (i.e. the amount released in each collected fraction divided by the tissue content at the time of collection) was calculated.

After some 30 min the rate of efflux of purines reached a steady level of 0.71 ± 0.03 (24) per cent of tissue content per min and after 70 min it was 0.74 ± 0.11. The basal rate of efflux was not affected by 1 μM morphine (0.78 ± 0.09), 1 μM naloxone (0.71 ± 0.09) or by morphine-naloxone (0.82 + 0.12). Between 7 and 50 min after start of superfusion veratridine (10 μM) was included in the buffer. As seen in Fig. 1 this caused the release of purines as well as noradrenaline. The release of noradrenaline was abolished by omission of calcium and inclusion of EGTA, while the purine release was more than 50% reduced. The purine compounds were inosine, adenosine and hypoxanthine, predominantly as judged by TLC. Interestingly the release of noradrenaline was much faster in onset than the release of purines (Fig. 1). This could indicate that the mechanism behind the release of the two types of compounds were different, for example that purine release is secondary to the release of a primary transmitter.

The depolarization-induced release of purines was not completely reproducible. During the first stimulation (47–50 min) 3.4 ± 0.4% of tissue content was released, during the second (87–90 min) 2.2 ± 0.7. Therefore the effect of drugs was tested during the second stimulation period (present from 65 min onwards) and the amount released during this second stimulation (S_2) was divided by that released during the first (S_1) in the same slices. It is seen in Fig. 2 that morphine (1 μM) caused a significant enhancement of the purine release. Naloxone (1 μM) had no effect, nor did the combination of morphine and naloxone.

Thus, the present results demonstrate that morphine increases veratridine induced purine release from rat cortical slices. The effect of morphine was not observed in the presence of

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Morphine increases depolarization induced purine release from rat cortical slices

By

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Theophylline antagonizes several different actions of morphine, including analgesia in mice (Ho *et al* 1973) the inhibitory effect of morphine on electrically induced contractions in the guinea pig longitudinal muscle myenteric plexus (Sawynok and Jhamandas 1976), the direct stimulatory effect of morphine (Grubb and Burks 1975), and the inhibitory effect of morphine on acetylcholine from brain slices (Jhamandas *et al* 1978) The mechanism of action of theophylline is not known However theophylline is known to antagonize several actions of adenosine. For example, the inhibitory effect of adenosine on field stimulated guinea-pig longitudinal muscle is inhibited by theophylline (Sawynok and Jhamandas 1976). Adenosine inhibits acetylcholine and noradrenaline release (Sawynok and Jhamandas 1976, Vizi and Knoll 1976 and this effect is also antagonized by theophylline.

Theophylline thus inhibits effects of both morphine and adenosine, but the two types of agents are not acting on the same receptors, since adenosine effects are not antagonized by naloxone which readily inhibits morphine effects (Sawynok and Jhamandas 1976, Gustafsson, Hedqvist and Fredholm, unpublished data) The data are, however compatible with the opinion that morphine induces release of adenosine (or a related compound) and that this accounts at least partly for the effects of morphine. Circumstantial support for such a scheme is provided by the report that the inhibitory effect of morphine on contractions of the field stimulated guinea-pig ileum is potentiated by dipyridamol (Gintzler and Musacchio 1975), a compound known to potentiate the effects of adenosine by inhibiting its inactivation.

We report the preliminary finding that morphine enhances depolarization induced release of adenosine and other purines from rat cortical slices.

After decapitation the rat brains were rapidly dissected out. Thin slices (0 5–1 mm) of the occipital cortex were cut by a razor blade From the middle of the slice reula section (7 mm diam) was punched out. 4 slices were incubated at 37°C in 5 ml Krebs-Ringer bicarbonate buffer for 40 min with 10 µCi 2,8- H adenine C-adenine or 1-7 H-noradrenaline (Radiochemical Centre, Amersham). O the average some 10 nCi was taken up per slice After washing, the slices were placed in thermostated plastic chambers and superfused with Krebs-Ringer bicarbonat buffer of the following composition (mM): NaCl (118), KCl (4.85), $CaCl_2$ (0.25), + $MgSO_4$ (1 15), KH_2PO (1 15), N HCO (25) glucose (11 1), pH 7.2 7 4 gassed with 95% O_2 and 5% CO_2.

Samples of the superfusion solution were continuously taken and the radioactivity counted in a sci til-lation coctail consisting of 4 g/l Omnifluor (Packard) n toluene Triton X 100 (2 1). At the end of the ex

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naloxone suggesting that it is exerted over morphine receptors. The mechanism behind the veratridine-induced release of purines is not known, therefore the mechanism behind the morphine effect remains obscure. Veratridine is known to depolarize nervous elements causing rapid changes in ion composition including a rise in internal calcium level (Blaustein 1975) There is also a rapid fall in ATP content (Li and White 1976). The release of purines may be a reflection of this fall in ATP content, and morphine may enhance purine release by enhancing ATP breakdown or diminishing its resynthesis. Morphine did not enhance spontaneous overflow indicating that whatever the mechanism may prove to be it would appear to be dependent upon nerve activity leading to depolarization.

Although the data are very preliminary it is interesting to speculate that increased levels of extracellular purines may contribute to the effects of morphine As noted in the introduction this may possibly explain the otherwise puzzling partial morphine antagonism by methylxanthines In view of the manifold effects of purines on nervous tissue, including increased cyclic AMP levels, decreased neuronal firing rate, and decreased transmitter release (Phillis 1977 Hedqvist and Fredholm 1976 Vizi and Knoll 1976) further studies along these lines are being carried out

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The International system of units (SI)

The following symbols and units, recommended by the SI are being used in Acta Physiologica Scandinav Certain units, not included in SI will still be permitted.

SI units with recommended symbols

Units	*Symbols*
kilogramme	kg
second, millisecond	s ms
mole, millimole, micromole, nanomole, picomole	mol mmol µmol nmol pmol
meter millimeter micrometer nanometer	m mm µm nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s^{-1})
newton (force)	N (kg m/s^2)
pascal (pressure)	Pa (N/m^2)
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m^2)

Conversion factors to be given in Methods.

Permitted non-SI units

Units	*Symbols*
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 Pa)
curie	Ci
liter milliliter microliter	l ml µl
degree Celsius	°C